Talanta 84 (2011) 480–486

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development and validation of a rapid HPLC method for the determination of five banned fat-soluble colorants in spices using a narrow-bore monolithic column

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ARTICLE INFO

Article history:
Received 21 September 2010
Received in revised form
29 December 2010
Accepted 16 January 2011
Available online 26 January 2011

Keywords:
Sudan I–IV
Para Red
High-performance liquid chromatography
FastGradient monolithic column

ABSTRACT

This work reports a fast and simple liquid chromatographic method for the simultaneous determination of five banned fat-soluble synthetic colorants, namely Sudan I–IV and Para-Red, in spice samples. The analytes were successfully separated isocratically in less than 5 min on the new narrow bore monolithic column, FastGradient® Chromolith (50 mm × 2.0 mm i.d.) using a mobile phase of 0.1% (v/v) HCOOH/acetonitrile (35/65%, v/v) at a flow rate of 1.5 mL min^{-1}. All colorants were detected at 506 nm. The main parameters (mobile phase composition, flow rate, injection volume) affecting the separation were studied. The proposed method was thoroughly validated in terms of linearity, LODs, precision and accuracy. The method was applied to the determination of the studied azo-dyes in various spices (paprika, chilli and mixed spice powders) after ultrasound-assisted extraction. Satisfactory recoveries, ranging from 92% to 109% were obtained.

1. Introduction

Food coloring is obtained by using natural or synthetic colorants. In food manufacturing processes, synthetic colorants are mainly used to restore or provide a desired color to a product because the natural colorants are less stable than synthetic ones under the food processing conditions [1,2]. Unfortunately, many of the synthetic colorants pose a potential risk to consumers' health if their daily intake exceeds the maximum permitted levels established by World Health Organization [3].

Sudan I–IV dyes (Fig. 1) are a group of synthetic colorants which have been classified as a possible human carcinogens [4]. Para Red is also an azo-dye structurally similar to Sudan I and it is also suspected by the Food Standards Agency (FSA) as genotoxic carcinogen [5]. Due to this fact their use in foodstuffs has been prohibited by the European Commission [6]. However, in some Eastern countries, these dyes are still used in order to intensify the red-orange color in spices. In this sense, accurate and reliable analytical methods for the quantification of these colorants in foods are needed.

Analytical methods reporting the determination of Sudan dyes in food samples have been reviewed quite recently [7]. Among analytical techniques, high performance liquid chromatography (HPLC) predominates this type of analysis since it offers the potential of multi-analytes screening, enhanced selectivity and sensitivity [8–27]. Since the Sudan and Para Red dyes (Fig. 1) are highly hydrophobic compounds (e.g. log $P_{(\text{Sudan I})}$ = 5.86 [28]) separation is typically carried out under reversed-phase conditions. Various detection modes include UV–Vis [8–13], photodiode array (PDA) [14–16], chemiluminescence (CL) [17], electrochemical (ED) [18] and mass spectrometry (MS) [19–27]. A more detailed overview of the previous reported HPLC (including chromatographic conditions, columns types, and analysis time) can be found in Table 1.

Fast-LC is a major trend in modern food analytical chemistry since there is a continuously growing demand for the quality control of large amounts of samples in the minimum possible time. As can be clearly seen in Table 1, only UPLC methods using sub-2 μm particulate columns fulfill this demand, offering analysis times in the range of 3–6 min [13,26,27]. The main "disadvantage" of using sub-2 μm columns is the need for upgrading/replacing the existing HPLC instrumentation to setups capable of operating in the range of 400–1000 bar. On the other hand, low/medium-pressure monolithic stationary phases reduce the analysis time via elevated flow rates (3–10 mL min^{-1}) increasing this way the consumption of mobile phase and wastes generation [29]. A hybrid solution has been proposed very recently by Merck with the commercialization of the new member of the Chromolith® monolithic columns

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doi:10.1016/j.talanta.2011.01.043

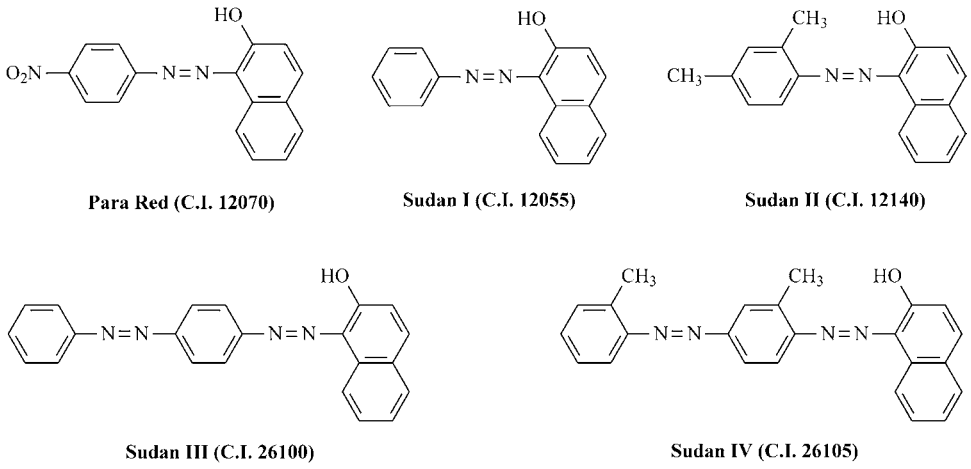

Fig. 1. Chemical structures and Color Index (C.I.) numbers of the studied azo-dyes.

family, namely the FastGradient® column [30]. This new narrow-bore monolithic column (2.0 mm i.d. × 50 mm length) provides high performance separations at very low operating pressures. This unique feature makes the column compatible not only to UPLC instruments but to conventional HPLC setups as well offering an interesting hybrid solution. Additionally, the typical working flow rates of 0.2–1.0 mL min^{-1} are ideal for mass spectrometric detection.

The aim of this work was the development and validation of a new rapid HPLC method for the isocratic separation and determination of five fat-soluble banned colorants in various spices samples using a narrow-bore monolithic column. To best of our knowledge this is the first report on the use of a monolithic stationary phase for this type of separation/analysis. The proposed assay is isocratic, faster than typical HPLC methods using particulate columns and comparable to UPLC approaches (Table 1). Validation was carried out in different spice food matrices and the method was successfully applied to the analysis of various real food samples.

2. Experimental

2.1. Chemicals, solutions and materials

Sudan I (1-(phenylazo)-2-naphthol), Sudan II (1-[(2,4-dimethylphenyl)azo]-2-naphthalenol), Sudan III (1-[4-(phenylazo)phenylazo]-2-naphthol and Sudan IV (1-[[2-methyl-4-[(2-methylphenyl) azo]phenyl]azo]-2-naphthalenol) with purities > 96% and Para Red (1-(4-nitrophenylazo)-2-naphthol) (purity 95%) were all purchased by Sigma–Aldrich (Steinheim, Germany). HPLC grade acetonitrile (ACN) used in the mobile phase was provided by Panreac (Barcelona, Spain) and ultra-pure water was produced by a Millipore system Ultra Clear TWF UV (SG) (Barsbuttel, Germany). Organic solvents such as acetone and dichloromethane were of analytical-grade and provided by Merck (Darmstadt, Germany).

Individual standard stock solutions of the colorants (200 μg mL^{-1} each) were prepared in aluminum-wrapped containers by dissolving the appropriate amount in a mixture of

Table 1
Overview of HPLC methods for the determination of Sudan I–IV and Para Red in various foodstuffs.

Technique	Analytical column	Column dimensions[a]	Elution type	Flow rate (mL min^{-1})	Analysis time[b] (min)	References
HPLC-UV	C18 Lichrospher ODS	200 mm × 4.6 mm i.d., 5 μm	Isocratic	1.0	7	[8]
HPLC-UV	C18, Spheri 5	250 mm × 4.6 mm i.d., 5 μm	Isocratic	2.0	NM[c]	[9]
HPLC-UV	RP-C18 Phenomenex Gemini	150 mm × 3.0 mm i.d., 5 μm	Gradient	0.6	13	[10]
HPLC-UV	ACE C18	250 mm × 4.6 mm i.d., 5 μm	Isocratic	1.0	8.8	[11]
HPLC-UV	Agilent Eclipse XDB-C18	150 mm × 4.6 mm i.d., 5 μm	Gradient	0.8	23	[12]
UPLC-UV	Acquity UPLC BEH C18	50 mm × 2.1 mm i.d., 1.7 μm	Gradient	0.4	5.62	[13]
HPLC-PDA	Cosmosil 5C 18-AR	150 mm × 4.6 mm i.d., 5 μm	Gradient	1.0	NM	[14]
HPLC-PDA	RP-C18 Varian Microsorb-MV	150 mm × 4.6 mm i.d., 5 μm	Isocratic	1.0	22.3	[15]
HPLC-PDA	Lichrospher C18	250 mm × 4.6 mm i.d., 5 μm	Gradient	1.0	20.9	[16]
HPLC-CL	Nucleosil RP-C18	250 mm × 4.6 mm i.d., 5 μm	Isocratic	1.0	25.5	[17]
HPLC-EC	Inertsil ODS-3	NM	Isocratic	1.0	23	[18]
LC–MS	Nova-Pak	150 mm × 3.9 mm i.d., 4 μm	Isocratic	0.15	16.92	[19]
LC–MS	Varian C18	50 mm × 2.0 mm i.d., NM	Gradient	0.25	10	[20]
LC–MS	Purospher Star RP-18	125 mm × 3.0 mm i.d., 5 μm	Gradient	0.5	13.5	[21]
LC–MS	ODS-3	250 mm × 2.1 mm i.d., 5 μm	Gradient	0.3	30.8	[22]
LC–MS	Phenomenex Luna C18	150 mm × 2.0 mm i.d., 3 μm	Isocratic	0.2	17	[23]
LC–MS	Spherigel C18	250 mm × 4.6 mm i.d., 5 μm	Gradient	1.0	30.29	[24]
micro LC–MS	Symmetry C18 capillary column	150 mm × 0.32 mm i.d., 5 μm	Gradient	0.005	26.82	[25]
UPLC–MS	Acquity BEH C18	100 mm × 2.1 mm i.d., 1.7 μm	Gradient	NM	4.19	[26]
UPLC–MS	Acquity BEH C18	50 mm × 2.1 mm i.d., 1.7 μm	Isocratic	0.35	2.98	[27]
HPLC-UV	FastGradient® reversed phase monolithic column	50 mm × 2.0 mm i.d.	Isocratic	1.5	3.9	Proposed method

[a] Column length × diameter (mm), particle size (μm).
[b] Expressed as retention time of Sudan IV.
[c] Not mentioned.

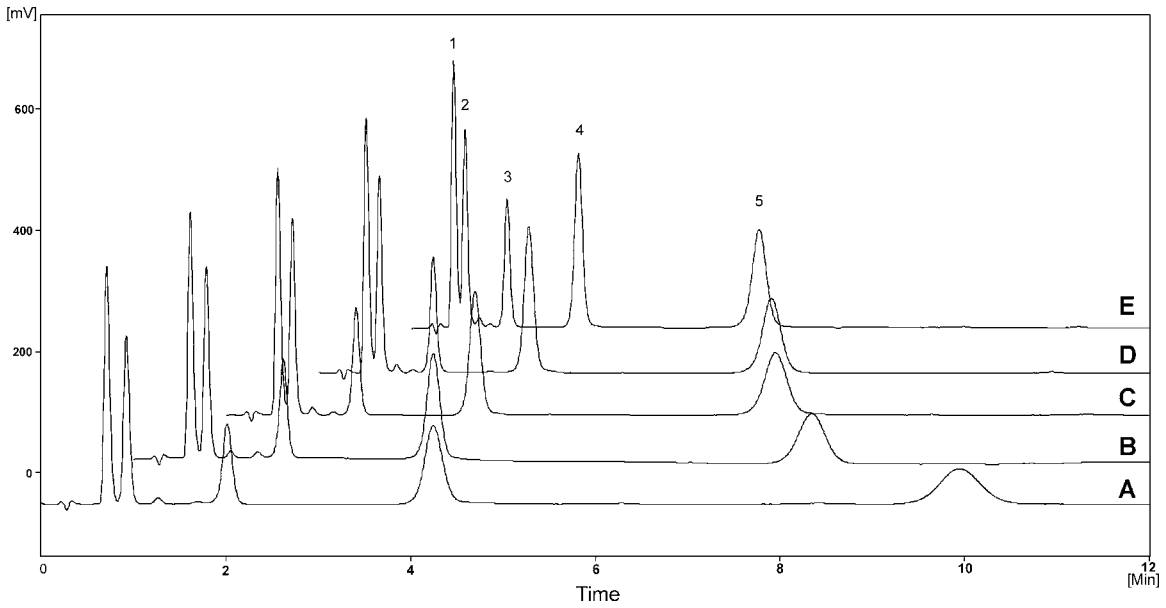

Fig. 2. Effect of the volume fraction of acetonitrile 60% (A), 63% (B), 65% (C), 67% (D) and 70% (E) on the separation efficiency of Para Red and Sudan I–IV: 1 = Para-Red; 2 = Sudan I; 3 = Sudan II; 4 = Sudan III; 5 = Sudan IV. Experimental conditions: flow rate: 1 mL min^{-1}; λ_{max} = 506 nm, T = 25 °C.

ACN/dichloromethane (50/50%, v/v). These solutions were stored at 4 °C. Working standard mixtures were prepared in ACN.

The acidic aqueous mobile phase (0.1%, v/v HCOOH/ACN, 35/65%, v/v) was filtered under vacuum through a membrane filter with a pore diameter 0.45 μm (Whatman®).

Nylon membrane disposable syringe filters (0.45 μm, Whatman®) were employed for sample filtration prior to injection into the monolithic column. Amber glass micro-vials (2 mL) were used as sample containers on the autosampler tray.

2.2. Equipment

Chromatographic analyses were carried out using a HPLC system equipped with a binary pump (LabAlliance Series 1500), an on-line vacuum degasser (Spark Holland BV, Emmen, Netherlands) and a Spectra System UV 2000 dual wavelength UV–Vis detector (ThermoFinnigan, USA). A Midas model 830 (Spark Holland BV, Emmen, Netherlands) autosampler was utilized for sample injection.

Chromatographic parameters (peak areas, retention times, theoretical plates, etc.) were calculated via the Clarity® software (DataApex, Czech Republic). Separations were performed on a FastGradient® reversed phase monolithic column (50 × 2.0 mm i.d., Chromolith, Merck). Polyether ether ketone (PEEK) tubing (0.18 mm i.d.) was used for all connections.

A Crison GLP 21+ pH-meter (Crison Instruments SA, Barselona, Spain) was employed for pH measurements. A Elma S 30 ultrasonic bath (Elma, Germany) and a Sigma 3–18 K centrifuge (Sigma Laborzentrifugen GmbH, Germany) was used for the pretreatment of solid samples.

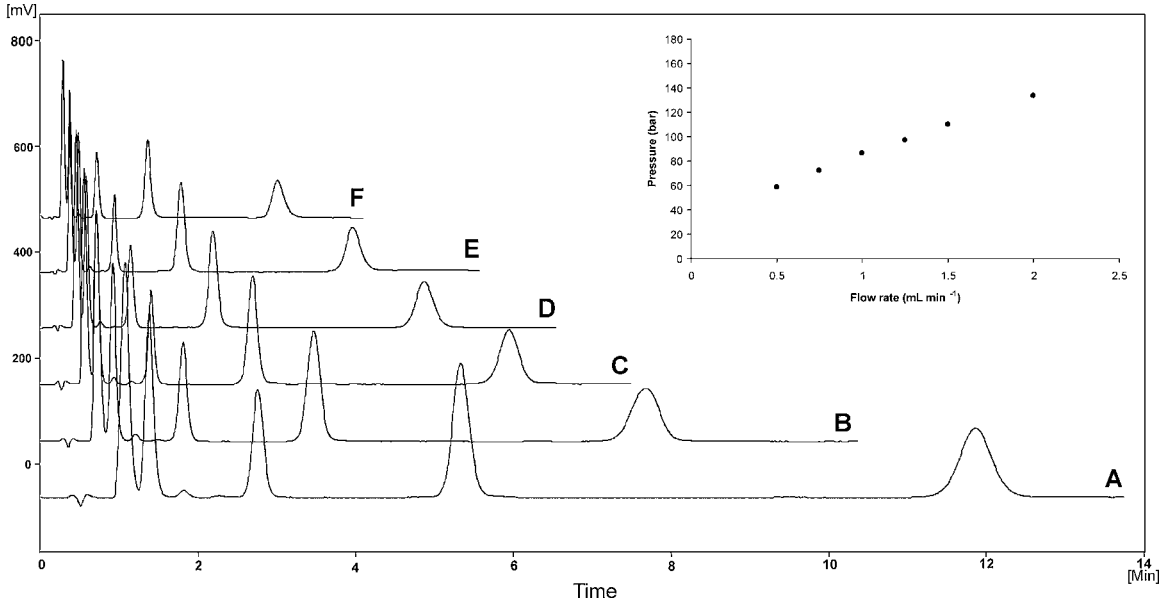

Fig. 3. Effect of the mobile phase flow rate 0.5 (A), 0.75 (B), 1.00 (C), 1.25 (D), 1.50 (E) and 2.00 (F) mL min^{-1} on the separation of the colorants and on the backpressure (inset). Experimental conditions: mobile phase: 0.1% (v/v) HCOOH/ACN, 35/65% (v/v); λ_{max} = 506 nm, T = 25 °C.

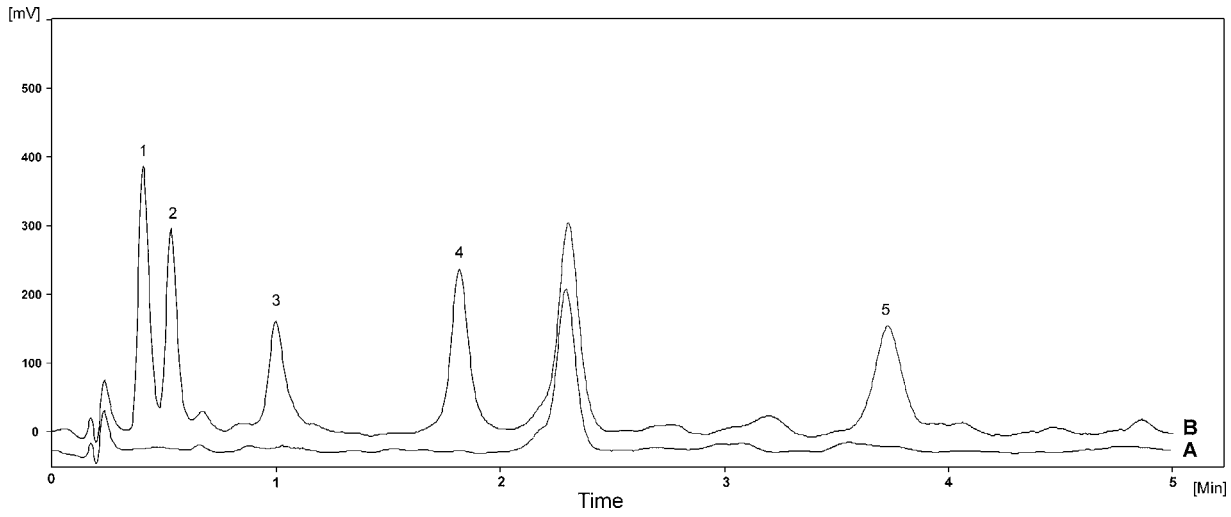

Fig. 4. Chromatograms of blank (A) and a spiked pooled spice sample (20 μg g^{-1} of each analyte) (B); peaks 1–5 as in Fig. 2.

2.3. HPLC conditions

An aliquot of 5 μL of standards or samples were injected into the monolithic column via the autosampler. The separation of the fat-soluble colorants was carried out isocratically using a mixture of 0.1% (v/v) HCOOH/ACN, 35/65% (v/v). The flow rate of the mobile phase was 1.5 mL min^{-1} while the detection of the analytes was performed at 506 nm. All experiments were carried out at room temperature. Under the above-mentioned conditions the analysis cycle was completed in less than 5 min. Peak area was used for signals evaluation, while each sample or standard was injected in triplicate.

It should be noted that in order to investigate and exclude the potential existence of late-eluted compounds, the first chromatogram of each real sample was monitored for 10 min.

2.4. Sample preparation

All real samples were obtained from the Greek market and included paprika powder (hot, sweet, biological cultivation), chilli powder and mixed spices powder.

Solid samples were ground into a mortar and pestle to a fine powder where necessary. 1.0 g of sample was accurately weighted into an amber glass vial wrapped with aluminum foil and dissolved in 10 mL of acetone/ACN 50/50% (v/v). The mixture was sonicated bath for 15 min to facilitate quantitative extraction of the colorants from the solid matrix. Subsequently, the supernatant was transferred to a conical bottom glass tube and centrifuged for 5 min at 5000 rpm. The resulting solution was filtered through a 0.45 μm Nylon syringe filter (Whatman®) and stored in the dark for 24 h prior to HPLC analysis (see discussion in Section 3.2). Three

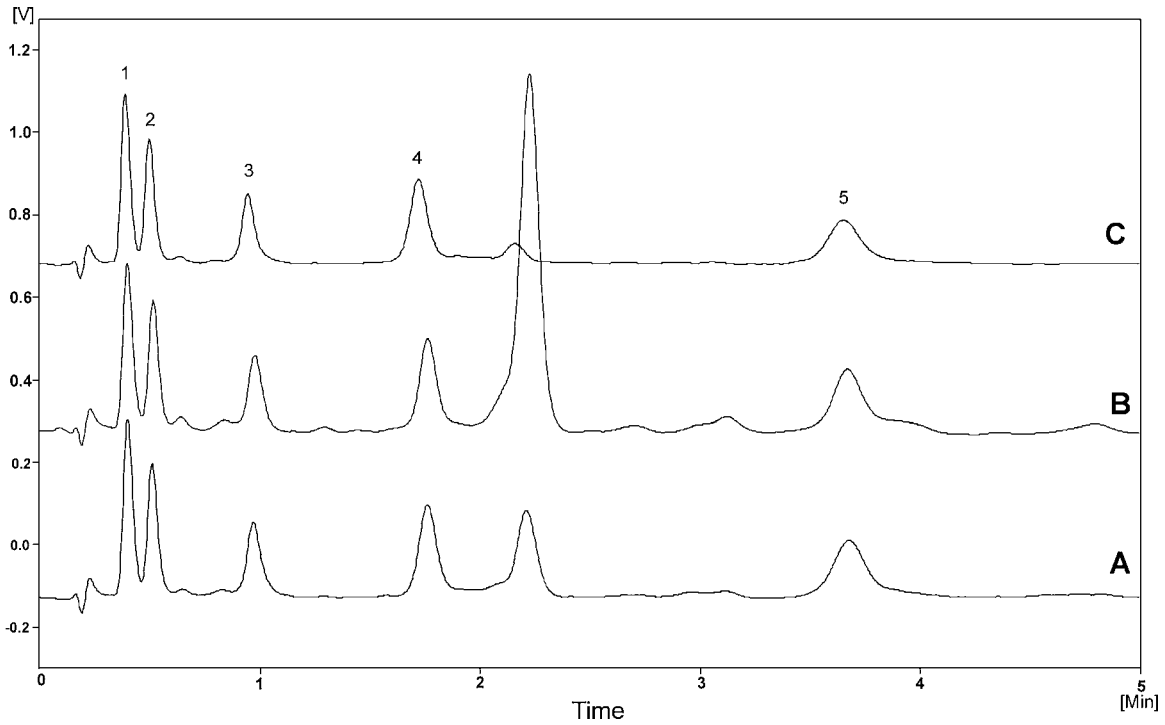

Fig. 5. Representative chromatograms of spiked spice samples at the 20 μg g^{-1} level of each analyte; chilli powder (A), mixed-spice (B) and paprika (C); peaks 1–5 as in Fig. 2.

Table 2
Analytical figures of merit of the proposed HPLC method.

Analyte	Matrix	Retention time (t_R, min)	Linear range ($\mu g\,L^{-1}$)	Regression equation $Y = S(\pm SDS)\ \gamma$ (analyte) $+ I$ ($\pm SDI$)[a]	LOD[b] ($\mu g\,L^{-1}/\mu g\,g^{-1}$)
Para Red	Standard	0.38	100–5000	$Y = 500(\pm 8) \times 10^{-3}\ \gamma$ [Para Red] $- 28(\pm 18) \times 10^{-3}$	35[c]
	Pooled spice	0.37	250–5000	$Y = 454(\pm 10) \times 10^{-3}\ \gamma$ [Para Red] $+ 70(\pm 32) \times 10^{-3}$	0.5[d]
Sudan I	Standard	0.48	200–5000	$Y = 383(\pm 6) \times 10^{-3}\ \gamma$ [Sudan I] $- 34(\pm 13) \times 10^{-3}$	50
	Pooled spice	0.47	500–5000	$Y = 354(\pm 4) \times 10^{-3}\ \gamma$ [Sudan I] $+ 57(\pm 13) \times 10^{-3}$	0.8
Sudan II	Standard	0.94	200–5000	$Y = 307(\pm 6) \times 10^{-3}\ \gamma$ [Sudan II] $- 27(\pm 15) \times 10^{-3}$	60
	Pooled spice	0.94	500–5000	$Y = 282(\pm 4) \times 10^{-3}\ \gamma$ [Sudan II] $+ 51(\pm 13) \times 10^{-3}$	1
Sudan III	Standard	1.78	200–5000	$Y = 596(\pm 5) \times 10^{-3}\ \gamma$ [Sudan III] $- 52(\pm 15) \times 10^{-3}$	70
	Pooled spice	1.77	500–5000	$Y = 553(\pm 9) \times 10^{-3}\ \gamma$ [Sudan III] $- 35(\pm 32) \times 10^{-3}$	1
Sudan IV	Standard	3.91	500–5000	$Y = 601(\pm 10) \times 10^{-3}\ \gamma$ [Sudan IV] $+ 96(\pm 63) \times 10^{-3}$	100
	Pooled spice	3.88	1000–5000	$Y = 562(\pm 14) \times 10^{-3}\ \gamma$ [Sudan IV] $- 25(\pm 46) \times 10^{-3}$	2

[a] Y is the peak area of each analyte ($n = 3$), γ (analyte) is the mass concentration of the analyte in $\mu g\,L^{-1}$, S is the slope and I the intercept, SDS and SDI the standard deviations of slope and intercept, respectively.
[b] The limit of detection was estimated by the S/N = 3 criterion.
[c] LOD in "aqueous" solutions (in $\mu g\,L^{-1}$).
[d] LOD in pooled sample (in $\mu g\,g^{-1}$).

sub-samples were individually prepared for each sample in all cases.

The fortified samples for recovery studies were prepared by spiking the sample powder with the appropriate amount of a standard mixture solution. After homogenization, the spiked samples were equilibrated for at least 3 h at 4 °C and protected from light until analysis.

3. Results and discussion

3.1. Study of chromatographic conditions

The aim of these experiments was to study the chromatographic behaviour of Sudan I–IV and Para Red using for the first time a reversed phase monolithic column. Among the commercially available monolithic columns, the new FastGradient column (50 mm × 2.0 mm i.d., Chromolith) was selected, as it offers the potential of developing fast-LC methods at low mobile phase consumption.

Due to the hydrophobic character of the analytes, organic solvent-rich mobile phases are typically used for their rapid elution under reversed phase conditions [7]. In a preliminary series of experiments mixtures of water with two common HPLC organic modifiers (ACN, methanol) were examined as mobile phases. The flow rate was set at 1 mL min^{-1} and the sample injection volume at 5 μL. The usage of ACN offered better peak symmetry and was therefore selected for subsequent studies.

The percentage of ACN in the mobile phase has the most profound effect on the separation efficiency of the analytes. Using a volume fraction of ACN of 70% (v/v) (or higher), the resolution (R_s) between Para Red and Sudan I was less than 1.32 while the analysis time did not exceed 4 min (Fig. 2). As expected, improved resolution was obtained with a gradual decrease of the percentage of ACN, achieving a satisfactory R_s between of the two analytes of 1.8 at 60% ACN. Furthermore, the separation efficiency (in terms of number of theoretical plates, N) of the less hydrophobic analytes (Para Red, Sudan I and II) was significantly enhanced while the N decreased moderately for the latter eluted compounds (Sudan III and IV). The best compromise in terms of resolution, efficiency and analysis time was obtained using a mobile phase containing 65% (v/v) ACN. The resolution factor between Para Red and Sudan I was 1.6 while the analysis time was quite satisfactory being 7 min (at a flow rate of 1.0 mL min^{-1}).

The studied azo-dyes are weak acids ($pK_{A(\text{sudan I\&II})} = 11.65$) since an inter-molecular hydrogen-bond could be formed with the phenolic hydroxylic groups [7]. On this basis, the impact of the mobile phase pH on the chromatographic behaviour of the analytes was investigated by acidifying the aqueous portion at values 3.0 and 4.5 by drop-wise addition of concentrated HCOOH. An average improvement in peak symmetry of 10% and in plate numbers of 80% was observed at pH 3.0 compared to un-acidified mobile phase.

Pressure drop along the FastGradient® column enables the usage of flow rates comparable or even higher to sub-2 μm UPLC columns but with conventional HPLC instrumentation. Typical chromatograms at flow rates in the range of 0.5–2.0 mL min^{-1} are depicted in Fig. 3. Using the flow rate of 1.5 mL min^{-1} the analysis was completed in less than 5 min ($P = 115$ bar). In all cases the resolution between of Para Red and Sudan I was not affected significantly (±3%) expect for 2 mL min^{-1} in which the variation was higher than 15%. Finally, the value of 1.5 mL min^{-1} was selected for subsequent studies since it offered a satisfactory analysis time of 5 min at reasonable low mobile phase consumption.

Column overloading is another parameter that has to be taken into account in Fast LC applications using narrow-bore columns [30,31]. The injection volume was investigated in the range of 1–5 μL using a mixture of 10 μg mL^{-1} of each colorant. The experimental results indicated that no practical variation in the resolution of the analytes and separation efficiency (data not shown). Based on these findings, an injection volume of 5 μL was chosen for subsequent experiments in terms of sensitivity.

3.2. Photo-stability of Sudan III and IV

There are literature data on the appearance of "fast-eluting" peaks in the HPLC chromatograms of Sudan III and IV [32,33]. A detailed recent study by Molder et al. using mass spectrometry proved that these peaks correspond to isomers of these azo-dyes originated by photochemically induced isomerization. This phenomena can lead to up to 10% quantitative errors (underestimation of Sudan III and IV) if the lightning conditions are not controlled. However, the photo-induced isomerization is – according to Molder – reversible when the compounds are stored in the dark for a sufficient time (3 h) [33].

The existence of these peaks was verified experimentally by our method by analysis of individual standards of Sudan III & IV at the 10 mg L^{-1} level under the selected HPLC conditions. The retention times of the "fast eluting" isomers peaks were 0.398 and 0.622 min, respectively. Our experiments confirmed the findings of Molder et al. since a considerable increase of the peaks area of the isomers was observed upon standing on the tray of the autosampler without protection from the light. On the other hand, relative areas of less than 2% were obtained when the vials were wrapped with aluminum foil. It should be noted that when analyzing a mixture of all colorants only the isomer of Sudan IV is resolved ($R_t = 0.622$ min) since the isomer of Sudan III is co-eluted with Para-Red ($R_t = 0.376$ min). Under controlled lightning conditions (see

Table 3
Within and day-to-day precision and accuracy of the proposed method in pooled spices sample.

Analyte	Fortified concentration ($\mu g\,g^{-1}$)	Within-day[a]		Between-day[b]	
		Recovery (%)[c]	RSD (%)	Recovery (%)[c]	RSD (%)
Para Red	10	92	2.3	91	7.1
	25	104	1.9	96	3.8
	50	103	3.1	97	3.7
Sudan I	10	105	3.5	94	5.6
	25	98	2.8	104	3.4
	50	101	2.6	105	4.3
Sudan II	10	103	2.4	109	6.2
	25	104	1.5	95	3.3
	50	96	1.6	96	4.1
Sudan III	10	95	2.2	107	4.7
	25	102	2.5	94	4.2
	50	102	1.8	103	3.6
Sudan IV	10	106	3.0	90	5.3
	25	103	1.7	93	4.9
	50	99	1.2	97	2.1

[a] Calculated for seven repeated analyses ($n = 7$).
[b] Studied for five consecutive days ($n = 5$).
[c] Calculated using the matrix-matched regression line.

Section 2.4), these peaks do not cause significant overestimation of Para-Red or underestimation of Sudan III & IV, and attempts to separate Para-Red and the isomer of Sudan III were therefore not made in terms of simplicity (isocratic elution).

3.3. Method validation

The proposed HPLC-UV method for the determination of Sudan I–IV and Para Red was validated in terms of linearity, limits of detection, within and between-day precision, accuracy.

3.3.1. Linearity, limits of detection and quantitation

The linearity of the proposed assay was validated in the range of 100–5000 $\mu g\,L^{-1}$ for each azo-colorant, using seven calibration points ($n = 7$) in both standard solutions and pooled spice matrixes ($n = 5$). The pooled sample was prepared by mixing and homogenization of 1.0 g of each individual spice. Further pretreatment of the pooled sample was carried out according to the procedure described in Section 2.4. Preliminary analysis of the pooled sample matrix shown that it is free from the studied azo-dyes and can therefore be used for validation purposes.

Regression equations, correlation coefficients, standard deviations of slopes and intercepts, LODs, and retention times of the analytes in "aqueous" and sample matrixes are listed in Table 2. The validity of the regression lines was confirmed by the residuals approach. In all cases the percent residuals were distributed randomly around the "zero" line, while they ranged between −1.62 and +1.20% for Para Red, −1.43 and +1.17% for Sudan I, −1.60 and +1.11% for Sudan II, −0.67 and +1.44% for Sudan III, −1.19 and +1.15% for Sudan IV.

Detection limits were calculated in the pooled sample matrix (in $\mu g\,g^{-1}$, Table 2) using progressively lower concentrations of the azo-dyes at a signal/noise ratio of 3/1 (S/N = 3). Each analysis was performed in triplicate.

3.3.2. Accuracy and precision of the method

The accuracy and precision of the proposed method were also validated in the pooled spices sample. The repeatability (within-day precision) and the intermediate precision (between-day precision) of the proposed method was evaluated by analysis of blank pooled spice sample spiked with 1 $\mu g\,mL^{-1}$ (low level corresponding to 10 $\mu g\,g^{-1}$), 2.5 $\mu g\,mL^{-1}$ (middle level corresponding to 25 $\mu g\,g^{-1}$) and 5 $\mu g\,mL^{-1}$ (upper level corresponding to 50 $\mu g\,g^{-1}$) of each azo-dye. The within-day precision was assessed by performing seven repeated injections of the three concentration levels during the day. The intermediate precision was established by analyzing the three synthetic samples, at a period of five consecutive days ($n = 5$). The accuracy was expressed in terms of dye recovery from the spiked pooled spice sample calculated by the matrix-matched regression lines. The results are summarized in Table 3. The recoveries were satisfactory in all sample tested and ranged between 90% and 109%. The R.S.D values varied from 1.2% to 3.5% and 2.1% to 7.1% for the within- and between-day for all analytes. The RSDs of the retention times were less than 1.0% for all analytes and concentration levels. A representative chromatogram of the analysis of a blank and a spiked pooled spice sample (20 $\mu g\,g^{-1}$ of each analyte) is illustrated in Fig. 4.

3.4. Extraction efficiency

Simple and effective sample pretreatment protocols for the extraction of the studied azo-dyes from solid or oil-based foodstuffs involve ultrasound-assisted extraction of the solid matrix with mixtures of ACN with other organic solvents (e.g. methanol, ethanol, acetone, etc.) [7]. In our study, the extraction recovery was investigated using different mixture of ACN with acetone, methanol and dichloromethane at volume fraction of 50/50% (v/v) for each solvent. As an indicator of extraction efficiency, the slopes of matrix-matched calibration curves (10–50 $\mu g\,g^{-1}$, $n = 5$) for the three extraction protocols were compared to the slopes of the "aqueous" calibration curves. Lower recoveries (77–82%) were obtained using ACN/methanol while comparable results (90 ± 5%) were achieved using either ACN/acetone or ACN/dichloromethane. Finally, the mixture of ACN/acetone (50/50%, v/v) was preferred for the extraction of the analytes due to the compatibility of acetone with the HPLC instead of dichloromethane. The experimental slopes for the ACN/acetone extraction protocol can be found in Table 2.

3.5. Analysis of real samples

The proposed method was applied to a variety of real spice samples, including three paprika powders (hot, sweet, biological cultivation), three chilli and two mixed spices powders available from local stores. Among the samples examined none of them was found positive on the colorants studied.

Matrix-effect was evaluated by analyzing each of the above mentioned samples spiked at three concentration levels (10, 25 and 50 $\mu g\,g^{-1}$) with the analytes. Quantification was carried out using

Table 4
Analysis of spice samples.

Sample	Fortified concentration ($\mu g\,g^{-1}$)	Recovery[a] (%)				
		Para Red	Sudan I	Sudan II	Sudan III	Sudan IV
Paprika powder (hot)	10	105	107	109	106	98
	25	98	97	102	95	101
	50	107	104	96	95	106
Paprika powder (sweet)	10	105	102	97	103	95
	25	93	94	95	105	102
	50	103	98	94	97	101
Paprika powder (BC)[b]	10	94	98	104	96	105
	25	103	102	95	103	103
	50	104	96	95	101	97
Chilli powder A	10	103	99	93	107	93
	25	92	93	98	103	97
	50	104	103	97	96	99
Chilli powder B	10	94	106	105	96	96
	25	103	99	104	104	98
	50	104	103	97	101	95
Mixed spice powder A	10	108	93	96	98	102
	25	96	98	104	106	105
	50	102	103	105	101	102
Mixed spice powder B	10	106	103	94	103	96
	25	93	99	106	97	101
	50	95	104	98	100	104

[a] Recovery calculated using matrix-matched calibration curves.
[b] Biological cultivation.

the "matrix-matched" curve in the pooled sample. The experimental results of Table 4 indicated that the recovery of spiked samples was varied from 93 to 109% in paprika powder, 92 to 107% in chilli powder and 93 to 108% in mixed spice powders. Fig. 5 depicts typical chromatograms of spiked chilli (A), mixed-spice (B) and paprika (C) samples. As it can be observed no peak from endogenous compounds interfere the peaks of interest.

4. Conclusions

An efficient, simple and fast analytical method for the simultaneous determination of Sudan I–IV and Para Red banned in European Union market food colorants by liquid chromatography-UV/Vis detection was demonstrated. The proposed HPLC methodology uses the novel FastGradient narrow-bore monolithic column for the isocratic separation of the analytes in less than 5 min at a flow rate of 1.5 mL min^{-1} and is the first report on the separation of this class of compounds using a monolithic column. Using conventional HPLC instrumentation the achieved analysis is comparable and in some cases even lower than previously reported UPLC approaches. The method allows the detection of the studied fat-soluble colorants at low concentrations (LODs: 0.5–2 $\mu g\,g^{-1}$) and is suitable for the screening of food samples according to international legislation directives.

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