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On-line SPE–UHPLC method using fused core columns for extraction and separation of nine illegal dyes in chilli-containing spices

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ABSTRACT

The presented work describes the development of a simple, fast and effective on-line SPE–UHPLC–UV/vis method using fused core particle columns for extraction, separation and quantitative analysis of the nine illegal dyes, most frequently found in chilli-containing spices. The red dyes Sudan I–IV, Sudan Red 7B, Sudan Red G, Sudan Orange G, Para Red, and Methyl Red were separated and analyzed in less than 9 min without labor-consuming pretreatment procedure. The chromatographic separation was performed on Ascentis Express RP-Amide column with gradient elution using mixture of acetonitrile and water, as a mobile phase at a flow rate of 1.0 mL min^{-1} and 55 °C of temperature. As SPE sorbent for cleanup and pre-concentration of illegal dyes short guard fused core column Ascentis Express F5 was used. The applicability of proposed method was proven for three different chilli-containing commercial samples. Recoveries for all compounds were between 90% and 108% and relative standard deviation ranged from 1% to 4% for within- and from 2% to 6% for between-day. Limits of detection showed lower values than required by European Union regulations and were in the range of 3.3–10.3 μg L^{-1} for standard solutions, 5.6–235.6 μg kg^{-1} for chilli-containing spices.

1. Introduction

About 90% of information transmitted to the human's mind is visual, and the attractive color of food is the essential part of decision about its quality, freshness, and therefore, health benefits.

For food coloring natural or synthetic dyes are available, where in manufacturing processes, the preference is given to the last due to the higher stability [1]. The red dyes Sudan I–IV, Sudan Red 7B, Sudan Red G, Sudan Orange G, Para Red, and Methyl Red are fat-soluble colorants legally used in industrial applications for coloring oils, plastics, soaps, waxes etc. and in research area as indicators [2]. It is well-known that many azo dyes are defined as genotoxic and/or carcinogenic, according to the European Food Safety Authority and therefore they are illegal for food and beverage usage in the most of the countries, including in the European Union [3]. The European Commission has designated the limit of detection for common HPLC method as 0.5–1 μg g^{-1} or 500–1000 μg kg^{-1}; furthermore it was pointed out that the presence of any banned dyes in food would be unacceptable in any level [3]. In spite of it sometimes these compounds are added illegally to products, particularly to products containing chilli, because of intensive and attractive red–orange color, low cost and wide availability [4]. For these reasons a quality control of food suspected of illegal use of Sudan red dyes is required and new, fast, modern, sensitive and reliable analytical methodologies for their identification and quantification must be developed.

As usual the determination of illegal dyes involves complex matrix removal and determination of contaminants at low concentrations. Therefore the pretreatment steps such as liquid–solid or liquid–liquid extraction [1,5], solid phase extraction (SPE) [2,6,7], pressurized liquid extraction [8], extraction with molecularly imprinted polymers [7,9,10] or cloud point extraction [11] for preconcentration and cleanup are required. Off-line SPE is the most often used method, but the large-sample volume, followed by additional procedures such as evaporation, reconstitution and therefore lower accuracy are expected. Also these methods can be labor-intensive, time-consuming and involve increased consumption of organic solvents, what may affect the recovery of the dyes and contribute highly to the total cost of the analysis. Consequently, one of the major scientific challenges in dyes analysis is to succeed higher sensitivity and selectivity while decreasing the organic solvent consumption. In this context, the on-line SPE offers a series of advantages such as development of faster methods with reduced consumption of solvents and less number of steps in sample preparation. Considering the above, several

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papers were published using on-line SPE in food analysis [12], but only a few described the application for the determination of dyes [6,7]. Unfortunately the mentioned methods were devoted to analyze relatively low number of dyes – Sudan I–IV – with separation time of about 20–25 min.

The presented contribution describes the development of simple, fast and effective UHPLC–UV/vis method using fused core particle columns for on-line SPE and separation of nine illegal dyes that are most frequently found in chilli-containing spices. Although on-line SPE–HPLC technique is well known in the field of chromatography, to our knowledge, coupling of the new types of fused-core particle columns has been described for on-line sample extraction [13] and analyte separation [13–17] in column switching chromatography systems in very few cases. Therefore, in the present paper we describe for the first time UHPLC on-line SPE column switching technique with fused-core column technology in both dimensions (extraction and separation). It is known that fused core particle technology allows to reduce mass transfer and to increase peak efficiency. Along with the improvement in peak efficiency, fused-core particles offer higher resolution, shorter analysis times, and lower flow resistance compared to traditional HPLC particles with 3–5 μm diameters. This approach is advantageous for analytes separation; moreover short fused core guard columns or precolumns are found to be very suitable for rapid sample extraction at relatively high loading flow rates in UHPLC systems. Compared to sub-2-micron particles used in UHPLC system, fused-core particle columns show similar efficiency of separation process and pressure limits in the UHPLC system are lower. Therefore the idea of on-line coupling of fused core sorbents to conventional HPLC or UHPLC instruments shows a novel and promising approach for instrumental applications in column switching systems. On-line coupled extraction techniques show further clear advantages compared with off-line SPE procedures, such as improved method precision and reproducibility, lower possibility of sample contamination and smaller sample volume requirements, as well as real higher sample throughput of the method, including the entire sample pretreatment process. The aim of the paper is to present a new approach for easy sample preparation and determination of illegal dyes employing modern fused-core particle columns coupled to on-line SPE–UHPLC system. The developed method was validated and successfully applied for the determination of Sudan red dyes in different chilli-containing samples.

From 2005 to the present day European Commission was finding unauthorized colors in food originated mostly from non-European countries [18]. Three different real samples from several countries were chosen for the study of the method applicability. The first is “Chilli Sauce Hot ABC” produced in Indonesia and it is one of the most common chilli-containing sauce in the local market. The second is “Feferony” is a pasta from chilli pepper which is produced in Slovakia and often used for preparing a homemade food. The third is powdered chilli-containing spice “Mojo” produced in Spain and it is a kind of popular typical Canarian spice. Hereby we tried to cover the whole variety of chilli-containing products with different consistencies such as sauce, pasta and dry powdered spice to study the reliability of the developed method and show the possibility of using the universal sample pre-treatment step.

2. Experimental

2.1. Chemicals

2.1.1. Chemicals and reagents

HPLC-grade solvents used were acetonitrile (ACN), tetrahydrofurane (THF) supplied by Sigma-Aldrich (St. Louis, USA), methanol was supplied by Lach-Ner (Neratovice, Czech Republic). All reagents used were of analytical grade or better. Ultra-pure water was obtained using Milli-Q Millipore System containing Millipak 40 sterile filter with 0.22 μm pore.

Solid standards of Sudan I (97%), Sudan II (90%), Sudan III (90%), Sudan IV (80%), Sudan Red 7B (95%), Sudan Orange G (97%), Methyl Red (90%), Para Red (95%), and Sudan Red G (90%) were purchased from Sigma Aldrich.

2.1.2. Characteristic of compounds

The studied dyes have molecular structures that are characterized by the nitrogen-to-nitrogen double azo bond between aromatic groups as depicted in Fig. 1. Their relatively high molecular weights and low polarity provide these dyes with highly hydrophobic properties (log *P* in the range of 5.0–8.5 [18]). In the structure of Methyl Red the phenyl part with carboxylic group renders this compound with higher polarity as well as free hydroxyl groups in Sudan Orange G structure with log *P* 3.31 and

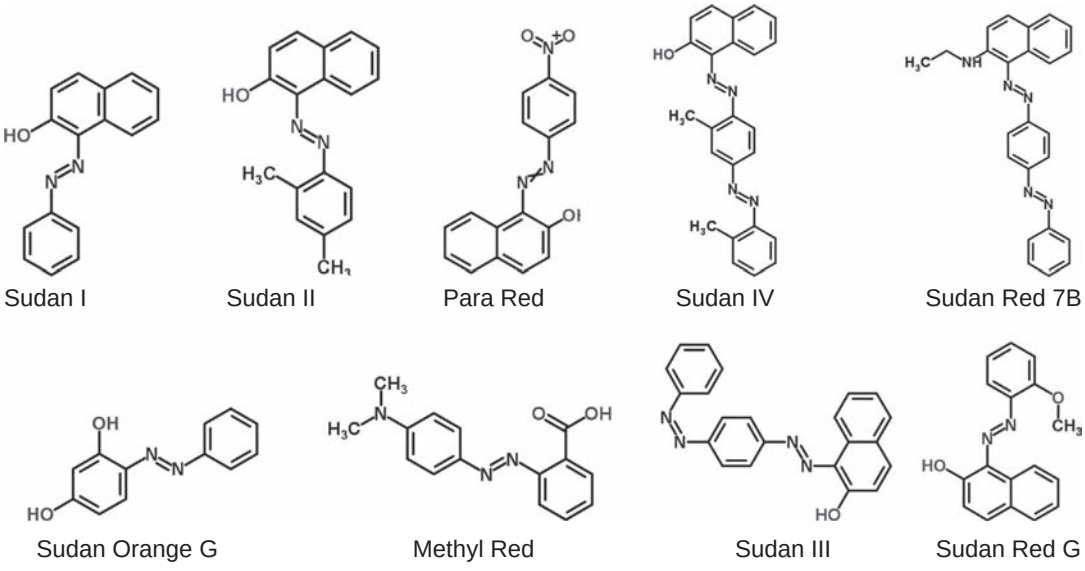

Fig. 1. Molecular structure of the studied illegal dyes.

3.7 [18], respectively. This was paid attention during the cleanup procedure when the matrix was flushed with water mobile phase.

2.1.3. Characteristic of samples

Three commercial samples such as two chilli-sauces "Chilli Sauce Hot ABC", "Feferony" and mixed powdered chilli-containing spice "Mojo" having different compositions and consistencies from several countries (Indonesia, Slovakia, and Spain) were purchased in the local market. Sauces were stored in the refrigerator at 4 °C. Before using, samples were mixed well.

2.2. Instrumentation and chromatographic conditions

The UHPLC system Thermo Scientific Dionex UltiMate™ 3000 equipped with a dual gradient pump, an autosampler, Diode Array Detector and column oven was used. A chromatographic separation was achieved on Ascentis Express RP-Amide (100 mm × 4.6 mm, 2.7 μm) analytical column with precolumn Ascentis Express F5 (5 mm × 4.6 mm, 5 μm). Data acquisition and processing control were done with Thermo Scientific Dionex Chromeleon™ software. Acetonitrile used as mobile phase B and water, filtrated through 0.22 μm Millipore filter, as mobile phase A were delivered at a flow rate of 1.0 mL min^{-1} according to the following binary elution gradient program: 4.00 min 65% mobile phase B, 4.00–7.00 min 95% mobile phase B, 7.00–16.00 min 65% mobile phase B, and 16.00–22.00 min 65% mobile phase B. As the SPE column Guard Cartridge Ascentis Express F5 (5 mm × 4.6 mm, 5 μm) was used. The washing mobile phase for SPE column consisting of a mixture of acetonitrile:water, 10:90 (v/v), was pumped for 2 min at flow rate of 1.0 mL min^{-1}. Valve switch for transferring the preconcentrated sample zone from the extraction to the analytical column was set at 2nd min in the back-flush direction. An injection volume of 30 μL was used.

2.3. Preparation of standard solutions

A standard stock solution of 1000 mg $L^{-1}$was individually prepared of each dye in THF and was stored at 4 °C in a dark place until used. The mixed stock standard solutions were prepared by mixing aliquots of each of the standard solution. Mixed standard solution with concentration of 5000 μg L^{-1} was prepared by diluting mixed stock solution and was used for the system suitability test.

2.4. Sample preparation

1.0 g of each sample was accurately weighted into a glass tube with a screw cap followed by spiking of standard solutions and two-step ultrasound-assisted extraction. The optimal period of sonication was studied and selected depending on the matrix and diffusion rate (Section 3.1). The first sonication was made in the presence of 5 mL of pure THF. Afterwards, a portion of the supernatant was filtered through a syringe PTFE filter with 0.2 μm pores. The residue was re-extracted with the next 5 mL of pure THF, to improve the diffusion of the interested compounds. The supernatant was filtrated, mixed together with the first fraction and adjusted to 10 mL with THF. Blank solutions were prepared from appropriate real sample in the same way, excluding a compound-spiking step.

From that matrix stock solutions a range of matrix calibration solutions were prepared by serially diluting them with individual blank solutions from non-spiked real samples. For example, the matrix stock solution with a concentration of 10,000 μg kg^{-1}, prepared from spiked Chilli sauce, was used to prepare calibration solution with concentration of 20 μg kg^{-1} by diluting with blank solution, prepared from non-spiked Chilli sauce. Similarly, all calibration solutions were prepared from Feferony sauce and Mojo spice.

2.5. Method validation

For the evaluation of the suitability of the whole procedure for the determination and quantification of nine illegal dyes in chilli-containing samples the developed method was validated in accordance with the Guidance for Industry, Bioanalytical Method Validation from Food and Drug Administration [19]. For the method specificity estimate, spiked samples and blank solutions prepared from real samples by the same way were analyzed. Recovery and accuracy of the method were performed by spiking known concentrations of mixed standard solution at different concentration levels. The limit of detection (LOD) and limit of quantification (LOQ) were measured to give the signal–to-noise ratio as 3 and 10, respectively.

3. Results and discussion

3.1. Optimization of the extraction conditions

In order to optimize the experiment, significant factors affecting the extraction efficiency of dyes were studied in this work.

Chilli-containing products, free from Sudan's and other illegal dyes, spiked with known concentrations of studied colorants were used to find the optimal extraction conditions and test the applicability of the method. The selection of extraction solvent is an essential consideration in analysis of complex food samples. Many different extraction solvents were used in the earlier described experiments [4]. In the present work ACN, dimethyl sulfoxide, methanol, and THF were examined. The last was preferred to other solvents because the amount of co-extracted compounds from the chilli matrix was reduced. THF was found to extract the dyes efficiently from real samples. Extraction time is an important factor to be considered in order to obtain efficient extraction. The extraction was made with ultrasound assistance in two steps (Section 2.4). The sonication was done at the different periods to find out the optimal conditions for quantitative extraction of the colorants from different matrices. Generally, maximum extraction efficiency could be achieved when extraction equilibrium is reached. In this work, the influence of extraction time ranging from 10 to 40 min on the extraction efficiency was evaluated. The sufficient recoveries were obtained using the sonication during 30 min (sequentially 15 min) for semi-solid samples such as Chilli ABC and Feferony sauces and 10 min (sequentially 5 min) for solid sample such as chilli-containing spice Mojo (Fig. 2).

3.2. Optimization of the chromatographic separation conditions

The key chromatographic conditions, such as stationary phase, mobile phase composition, gradient program, flow rate, injection volume, and column temperature affecting retention, selectivity, and efficiency, were studied and optimized for the chromatographic separation of Sudan I–IV, Sudan Red 7B, Sudan Red G, Sudan Orange G, Para Red, and Methyl Red.

In the previously described works it was shown that the retention of Sudan's dyes in reversed-phase chromatography is strong due to the lipophilicity of these compounds. Mostly the C18 stationary phase from the different producers was used for the separation of Sudan I–IV and other dyes [4]. In our experiment C18, RP Amide, F5 fused core particle stationary phases and one monolithic C18 column were examined. The comparison between satisfactory separation of analytes and peak shapes was found

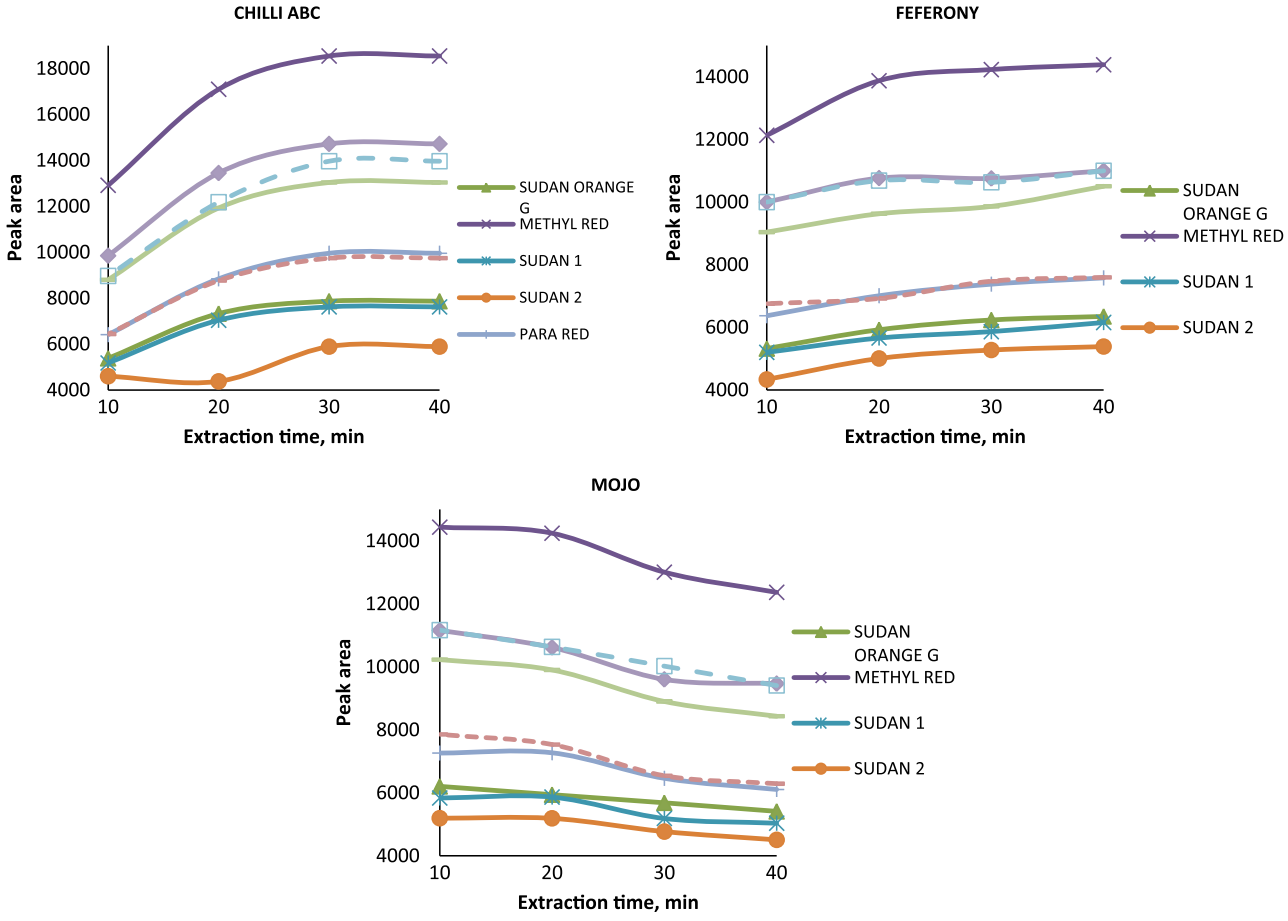

Fig. 2. Monitoring of ultrasound extraction of illegal dyes from different chilli-containing spiked samples.

using column Ascentis Express RP-Amide 100 × 4.6 mm with size of particles as 2.7 μm. To protect the analytical column during the procedure with complex real samples the precolumn was used. It was noticed that the stationary phase of precolumn can slightly affect the separation which was observed in the present work using Ascentis Express F5 to improve the separation of Sudan Red 7B and Sudan 4. As shown on the chromatogram, the resolution of these two peaks was critical and the different chemistry of the precolumn stationary phase enabled to improve the resolution of Sudan Red 7B and Sudan 4. The use of fused core particle precolumn for SPE brought a benefit such as a chemical and pressure stability, sufficient reproducibility and longer life-time than traditional SPE cartridges. The repeatability and recovery of obtained results were at a sufficient level after 400 injections.

Due to the hydrophobic character of most of the measured analytes, organic solvent-rich mobile phase is needed to be used for their rapid elution under reversed phase conditions [4]. The most frequently used organic solvents such as methanol and acetonitrile in a mixture with water were tested. The usage of acetonitrile showed better peak shapes and response of analytes. Consequently, as a mobile phase mixture of acetonitrile and water (from 65:35, v/v) was used. The volume content of acetonitrile largely influenced the efficiency of separation. The optimal percentage was found to be 65 for the separation of Sudan Red G from Sudan I and Sudan Red 7B from Sudan 4 with satisfactory resolutions of 2.51 and 2.13, respectively. The isocratic elution was tested initially with successful separation of five compounds and low response for Methyl Red, Sudan I and II. So, with the aim to improve separation the gradient elution was needed. The separation was investigated under different gradient elution conditions starting from 65% of acetonitrile. Using gradient elution conditions described in Section 2.2, nine studied colorants were baseline separated properly with symmetric peak shapes, and fast analysis has been achieved. The pressure was relatively low up to 20 MPa.

As additives formic acid and acetic acid were tested at different concentrations without significant improvement in separation or peak shapes, resulting in what was not used in the experiment. Flow rates in the range of 0.7–2.0 mL min^{-1} were examined. With low flow rates of 0.7 and 0.8 mL min^{-1} the peak asymmetry was unsatisfactory. On the other hand, higher flow rates of 1.5 and 2.0 mL min^{-1} allowed to reduce the analysis time but without baseline separation of all nine compounds. Considering the results, the flow rate of 1.0 mL min^{-1} which provided good separation in 9 min and peak asymmetry of 1.31 were used in the present work. The injection volume is important when the method for determination of illegal contaminants at low concentration is developed. With the aim to enhance the ability of the sensitivity of the method the injection volume in the range of 1–50 μL was studied. Certainly the developed method can be used with an injection volume of 1 μL but with lower sensitivity in comparison with 30 μL. The larger injection volume of 50 μL showed high sensitivity but accompanied by undesired faster elution of Methyl Red and Sudan Orange G from the precolumn, during sample loading, caused by the use of THF as a sample solvent, which has considerable eluotropic strength. Moreover, the injection of 50 μL provided complications associated with sample carry-over in the internal parts of the equipment.

The compromise in terms of sensitivity, SPE column overloading, sample consumption and easy operation was obtained

using the injection volume of 30 μL. The effects of temperature on the peak capacity, separation and time of analysis were studied. Lower column temperature in the range of 30–50 °C allowed to separate all compounds while the time of analysis was prolonged. Maximal peak heights of all compounds and sufficient resolution were found at the temperature of 55 °C. When the column oven temperature was 60 °C the elution of compounds from short SPE column was accelerated and compounds were flushed off with the matrix. For these reasons column temperature 55 °C was selected as an optimal for our purpose. The influence of increasing temperature on peak capacity was studied. Fig. 3 shows the effect of temperature on the peak capacity at the same flow rate 1 mL min^{-1} and time of gradient 12 min. For Sudan 3, Sudan Red 7B and Sudan 4, an increase in temperature always increased the peak capacity. On the contrary, for Methyl Red, Sudan Orange G, Para Red, Sudan Red G, and Sudan 1 and 2, higher temperature did not affect the peak capacity significantly. This behavior could be explained by the fact that more retained compounds, such as Sudan 3, Sudan Red 7B and Sudan 4, are more sensitive for increasing the temperature and lowering the viscosity of mobile phase than less retained analytes.

The signal of analytes can be monitored at their individual maximum absorbance wavelengths. However in the present work colorants were divided into two groups: first was detected at wavelength of 500 nm and the second group included Sudan Orange G which was detected at 420 nm.

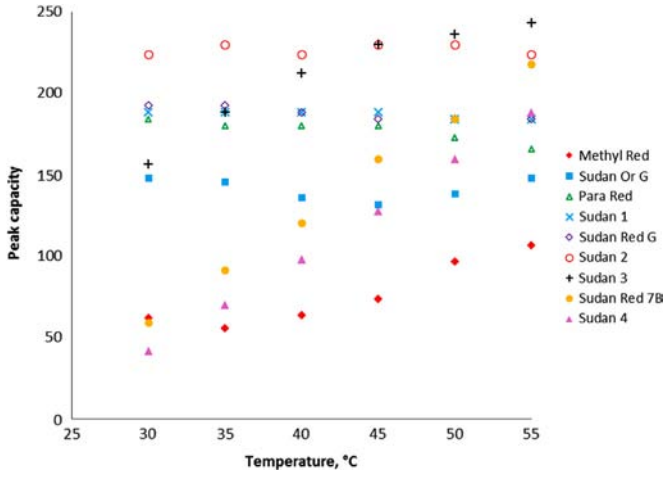

Fig. 3. Influence of column temperature on the peak capacity.

3.3. On-line SPE: optimization of cleanup procedure

Since in food analysis the contaminants are found at very low concentration levels in complicated matrix, a preconcentration and cleanup step is necessary. Fig. 4 shows the setup for on-line SPE–UHPLC. In the first step (a), the sample was injected to the SPE column with the help of the autosampler and the first pump. The sample matrix, containing more polar compounds and impurities, was rinsed out to the waste, by 10% of ACN at a flow rate of 1.0 mL min^{-1} within 2 min, while the analytes of interest were concentrated on the SPE column. At the same time the analytical column was equilibrated by the mobile phase from the second pump. When the matrix was washed out from SPE column, the six-port valve was switched (b) to couple with the analytical column. This step involved the elution of analytes from SPE column in the back-flush direction followed by separation on analytical column. After this transfer, the valve was switched back to the initial position (a). The SPE column was washed and equilibrated using the flow from the first pump while the separation on analytical column continued running.

Influence of stationary phase of the SPE column was studied. It was noted that the stationary phase affected the peak shapes and the combination of different stationary phases of SPE and analytical column could offer more possibilities for the separation improvement. In the present work the following combinations of SPE and analytical column were examined: RP Amide–RP Amide, F5–F5, C18–RP Amide, RP Amide–F5, and F5–RP Amide. The last combination showed the best separation efficiency and better peak shape of Methyl Red and Sudan Orange G. Concentrations of 5%, 10% and 20% of ACN in water were tested for SPE cleanup procedure. The maximum of peak height was obtained using 10% of ACN which allowed to fully elute the components from the SPE column without further contamination. Using higher concentration of ACN influenced the peak symmetry of Methyl Red and showed lower signal. The time for on-line SPE cleanup step is defined taking into account not to lose the analytes of interest. Due to the higher polarity of Methyl Red the sticking point was its early elution from SPE column. In the present work, it was found that 2 min is the optimal period to flush enough of the matrix and to retain all analytes. The second switch of valve was done precisely

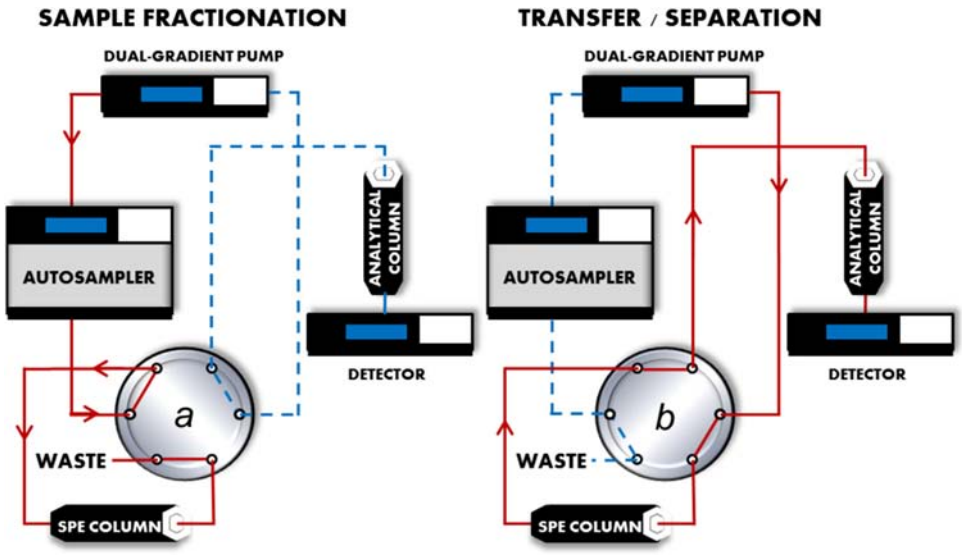

Fig. 4. Scheme of on-line SPE–UHPLC system (valve position (a) – on-line extraction; valve position (b) – separation of extracted dyes).

Table 1
System suitability test results.

Analyte	λ (nm)	t_R [a]	Repeatability		A_S [b]	R_S [c]	P_c [d]
			t_R, R.S.D. (%)	Peak area, R.S.D. (%)			
Methyl Red	500	4.3	0.33	1.95	0.78	-	119
Para Red	500	5.8	0.08	0.33	1.31	5.47	197
Sudan Red G	500	6.1	0.05	2.74	1.07	2.51	207
Sudan 1	500	6.3	0.06	3.14	1.11	16.99	212
Sudan 2	500	7.4	0.04	2.35	1.24	10.63	243
Sudan 3	500	8.0	0.03	2.40	1.26	9.77	251
Sudan Red 7B	500	8.6	0.03	1.99	1.2	2.13	212
Sudan 4	500	8.8	0.04	3.57	1.25	1.8	184
Sudan Orange G	420	4.9	0.05	1.17	1.24	1.6	176

[a] Retention time, min.
[b] Asymmetry factor.
[c] Resolution of peaks.
[d] Peak capacity.

Table 2
The analytical characteristic of the developed method (standard calibrations, detection and quantification limits).

Analyte	Linear range (μg L^{-1})	Regression equation	r	LOD (μg L^{-1})	LOQ (μg L^{-1})
Methyl Red	20–10,000	$y=0.4469x+0.005$	0.9997	3.3	11.0
Para Red	20–10,000	$y=0.9931x+0.0107$	0.9995	3.6	12.0
Sudan Red G	20–10,000	$y=0.8801x+0.0123$	0.9995	3.6	12.3
Sudan 1	20–10,000	$y=0.6356x+0.0172$	0.9997	4.6	15.5
Sudan 2	20–10,000	$y=0.6183x+0.0095$	0.9997	5.2	17.6
Sudan 3	20–10,000	$y=0.9756x+0.0147$	0.9999	5.8	19.6
Sudan Red 7B	50–10,000	$y=0.6383x-0.0016$	0.9999	10.3	34.6
Sudan 4	20–10,000	$y=0.8548x+0.0011$	0.9997	4.4	14.9
Sudan Orange G	50–10,000	$y=0.5243x+0.0129$	0.9997	7.9	26.3

when all compounds were transferred from the SPE to analytical column. It should be noted that the temperature of SPE column must be accounted. When the SPE column was placed into column oven with analytical column at 55 °C the reproducibility of the method was better which was caused by the constant temperature for both the steps: cleanup and separation.

3.4. Validation

The developed method has to be accurate and be able to identify contaminants with high selectivity. The analytical method evaluation including method selectivity, linearity, detection limits, quantification limits, and accuracy was carried out.

3.4.1. System suitability test

System suitability was determined from six replicate injections of the mixed standard solution (Section 2.3) before real sample analysis. The system suitability assessment for the developed method established the following parameters: repeatability of retention time (t_R) and peak area, asymmetry factor (A_S), resolution of peaks (R_S), and peak capacity (P_c) for gradient elution. All parameters maintained R.S.D. values lower than 1.0% (Table 1). All critical tested parameters met the acceptance criteria during measurements.

3.4.2. Linearity

In order to test the linearity, standard calibration curves and matrix calibration curves at six levels were prepared. Linearity of the proposed method was studied in the range of 20–10,000 μg L^{-1} for standard calibration solutions, and 20–10,000 μg kg^{-1} for matrix calibration solution, depending on the compound in standard solutions and spiked real samples. Preliminary studies showed that real samples matrices are free from measured compounds and can therefore be used for validation procedure. According to the values of the linear correlation coefficients for the matrix calibration curves all the compounds showed good correlation ($r \geq 0.999$). Regression equations, correlation coefficients and linear ranges are

Table 3
The analytical characteristic of the developed method (matrix calibrations, detection and quantification limits).

Analyte	Matrix	Linear range (μg kg^{-1})	Regression equation	r	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
Methyl Red	Chilli sauce	20–10,000	$y=0.5133x+0.0016$	0.9991	5.6	18.9
	Feferony	20–10,000	$y=0.6135x-0.0101$	0.9990	6.1	20.0
	Mojo	150–10,000	$y=0.6528x-0.027$	0.9983	44.8	149.6
Para Red	Chilli sauce	100–10,000	$y=1.053x-0.0017$	0.9995	24.3	81.7
	Feferony	200–10,000	$y=1.1152x-0.0088$	0.9994	58.5	196.3
	Mojo	400–10,000	$y=1.1791x-0.0154$	0.9999	107.2	359.2
Sudan Red G	Chilli sauce	150–10,000	$y=0.9138x-0.0036$	0.9998	36.1	120.3
	Feferony	100–10,000	$y=0.9345x-0.0057$	0.9995	20.3	67.4
	Mojo	100–10,000	$y=0.9047x-0.004$	0.9997	22.0	73.4
Sudan 1	Chilli sauce	50–10,000	$y=0.7075x-0.0007$	0.9997	12.5	41.9
	Feferony	50–10,000	$y=0.7447x-0.0043$	0.9991	9.6	32.2
	Mojo	200–10,000	$y=0.806x-0.0131$	0.9999	32.2	107.2
Sudan 2	Chilli sauce	100–10,000	$y=0.6363x-0.0028$	0.9997	31.8	100.4
	Feferony	100–10,000	$y=0.6543x-0.0028$	0.9997	21.2	70.9
	Mojo	50–10,000	$y=1.0591x-0.0126$	0.9999	14.6	48.8
Sudan 3	Chilli sauce	500–10,000	$y=0.9881x-0.0043$	0.9995	143.5	480.0
	Feferony	250–10,000	$y=0.995x-0.003$	0.9998	73.2	245.1
	Mojo	500–10,000	$y=1.0591x-0.0126$	0.9999	235.6	488.0
Sudan Red 7B	Chilli sauce	100–10,000	$y=0.6624x-0.0022$	0.9994	23.4	78.3
	Feferony	500–10,000	$y=0.6934x-0.0044$	0.9991	65.2	217.7
	Mojo	200–10,000	$y=0.6966x-0.0103$	0.9997	51.7	172.6
Sudan 4	Chilli sauce	50–10,000	$y=0.9253x-0.0046$	0.9991	11.2	37.5
	Feferony	200–10,000	$y=0.9417x-0.0064$	0.9994	47.6	159.3
	Mojo	50–10,000	$y=0.9674x-0.0151$	0.9994	8.7	29.1
Sudan Orange G	Chilli sauce	50–10,000	$y=0.5309x+0.0022$	0.9997	9.5	31.8
	Feferony	100–10,000	$y=0.5623x+0.0015$	0.9996	17.7	59.2
	Mojo	120–10,000	$y=0.5616x-0.0002$	0.9999	35.3	117.9

listed in Table 2 for standard calibration curves, and in Table 3 for matrix calibration curves.

3.4.3. Accuracy, precision, and selectivity

The accuracy and precision of the developed method were determined in spiked samples fortified at three concentration levels: 1000, 2500 and 5000 μg kg^{-1}. The within-day precision was measured by five repeated injections of three concentration levels during one day. The intermediate precision was established at a period of three days (n=5). The accuracy was expressed in terms of compounds recovery from the spiked samples. The data summarized in Table 4 indicated that method showed satisfied recoveries in the range of 94–104% for chilli sauce, 95–108% for Feferony sauce, and 90–108% for Mojo spices. R.S.D. values ranged from 1% to 4% for within- and from 2% to 6% for between-day for all analytes. The proposed method provided significantly better recovery and shorter time of separation compared to the previously described Matrix Solid Phase Dispersion Extraction–LC–DAD method for chilli-containing sauces by Enriquez-Gabeiras (recovery in the range of 48–99%, separation of Sudan I–IV in 25 min) [20]. The precision and recovery of developed method were similar to the described HPLC–DAD–ESI-MS method for the analysis of Sudan I–IV in chilli powder and chilli spices by Ma (recoveries ranged from 93.2% to 108.3%, R.S.D. less than 8.2%) [21]. These results demonstrated that the precision and accuracy of the proposed method were suitable for the routine monitoring purpose.

Colorants were successfully separated within 9 min and the resulting extracts were free from interferences at the retention time of the analytes. For selectivity, no interferences were detected in the corresponding retention times of target compounds by comparing chromatograms of spiked samples and blank samples. The results showed that the developed method has high selectivity for measured dyes (Fig. 5).

3.4.4. LOD and LOQ

Detection limits and quantification limits of investigated analytes were determined in standard solution and spiked samples as the minimum concentration giving a signal-to-noise ratio (S/N) of 3 and 10, respectively.

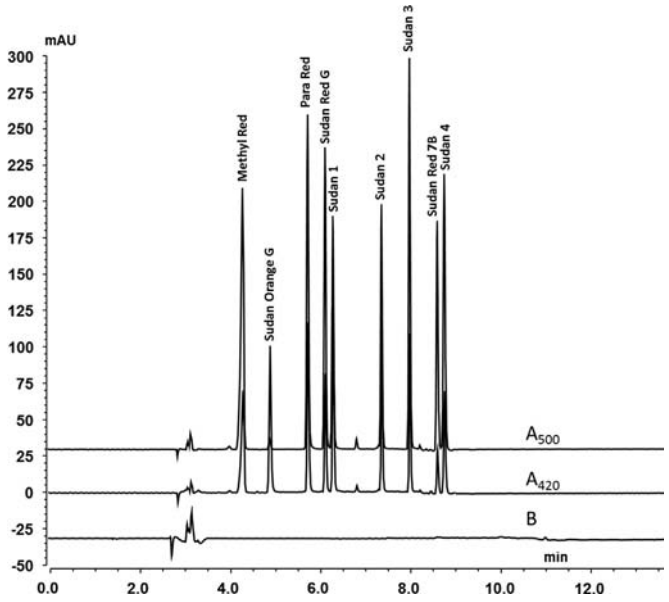

Fig. 5. On-line SPE–UHPLC chromatogram of spiked sample at 500 nm (A_{500}) and 420 nm (A_{420}) and blank extract (B) at 500 nm and 420 nm (overlaid).

Table 4
Accuracy and precision of on-line SPE–UHPLC method.

Compound	Fortified concentration (μg kg^{-1})	Within-day recovery (%) ± S.D.			Between-day recovery (%) ± S.D.		
		Chilli sauce	Feferony sauce	Mojo spices	Chilli sauce	Feferony sauce	Mojo spices
Methyl Red	1000	98 ± 3	101 ± 3	105 ± 2	104 ± 5	108 ± 6	107 ± 5
	2500	99 ± 4	98 ± 2	95 ± 2	101 ± 4	105 ± 3	102 ± 4
	5000	98 ± 3	97 ± 2	100 ± 2	100 ± 3	98 ± 4	95 ± 3
Para Red	1000	99 ± 3	99 ± 3	97 ± 3	102 ± 3	105 ± 3	106 ± 3
	2500	96 ± 3	97 ± 2	98 ± 2	101 ± 4	100 ± 4	101 ± 3
	5000	95 ± 3	97 ± 2	98 ± 3	97 ± 2	99 ± 4	99 ± 5
Sudan Red G	1000	101 ± 3	101 ± 4	99 ± 2	95 ± 5	108 ± 5	98 ± 4
	2500	100 ± 3	100 ± 2	96 ± 3	95 ± 5	104 ± 5	99 ± 4
	5000	94 ± 3	93 ± 3	99 ± 3	99 ± 6	99 ± 4	101 ± 4
Sudan 1	1000	94 ± 3	99 ± 4	100 ± 1	104 ± 6	103 ± 3	107 ± 6
	2500	101 ± 3	96 ± 3	101 ± 2	103 ± 4	97 ± 5	103 ± 3
	5000	99 ± 2	96 ± 2	98 ± 2	99 ± 4	96 ± 5	97 ± 4
Sudan 2	1000	98 ± 3	100 ± 4	107 ± 2	104 ± 5	100 ± 4	108 ± 4
	2500	98 ± 3	95 ± 3	96 ± 2	100 ± 6	98 ± 6	104 ± 3
	5000	98 ± 1	100 ± 3	101 ± 2	98 ± 3	98 ± 5	105 ± 4
Sudan 3	1000	99 ± 3	99 ± 1	98 ± 2	102 ± 5	101 ± 3	103 ± 2
	2500	96 ± 2	96 ± 1	97 ± 3	99 ± 3	100 ± 3	100 ± 4
	5000	99 ± 2	99 ± 3	100 ± 3	99 ± 6	93 ± 4	95 ± 5
Sudan Red 7B	1000	103 ± 3	103 ± 4	102 ± 4	104 ± 6	94 ± 4	93 ± 4
	2500	100 ± 4	101 ± 2	100 ± 1	104 ± 4	99 ± 5	98 ± 5
	5000	102 ± 2	99 ± 2	101 ± 3	96 ± 5	96 ± 3	90 ± 2
Sudan 4	1000	100 ± 2	98 ± 2	96 ± 3	104 ± 5	96 ± 5	94 ± 3
	2500	100 ± 1	102 ± 3	102 ± 1	102 ± 4	100 ± 4	93 ± 5
	5000	104 ± 2	103 ± 2	105 ± 2	102 ± 4	95 ± 3	95 ± 3
Sudan Orange G	1000	102 ± 3	102 ± 3	103 ± 2	101 ± 4	100 ± 3	99 ± 2
	2500	100 ± 4	99 ± 3	98 ± 3	100 ± 5	99 ± 3	99 ± 2
	5000	103 ± 1	103 ± 3	102 ± 4	98 ± 6	96 ± 6	95 ± 5

Table 5
Chromatography methods with UV/vis detection for the determination of Sudan's and other red-orange illegal dyes in chilli-containing food.

Method	Compounds	Samples	Sample preparation	Time of separation	Column	Linear range	LOD	Recovery	References
HPLC–DAD	Sudan I–IV	Chilli products	SPE	25 min	Agilent XDB-C18 (150 mm × 4.6 mm, 5 μm)	50–2500 μg L^{-1}	4.1–5.8 μg kg^{-1}	93.2–103%	[2]
HPLC–DAD	Sudan I–IV	Chilli products	Supramolecular solvent-based microextraction	71 min	Análisis Vínicos ODS (250 mm × 4.6 mm, 5 μm)	28–2000 μg kg^{-1}	2.7–7.4 μg kg^{-1}	86–108%	[23]
HPLC–UV	Sudan I–IV	Chilli powder	Cloud point extraction	22 min	Lichrospher C18 (250 mm × 4.6 mm, 5 μm)	10–500 μg kg^{-1}	2.0–4.0 μg kg^{-1}	80.7–85.45%	[11]
HPLC–UV	Sudan I–IV	Tomato sauce	On-line Molecularly imprinted polymers–SPE	20 min	Dikma Technologies C18 (250 mm × 4.6 mm)	5–1000 μg kg^{-1}	1.0–3.0 μg kg^{-1}	85.5%	[7]
HPLC–DAD	Sudan I–IV	Sauces and condiments	Matrix solid phase dispersion extraction	11 min	Phenomenex Synergy Polar RP (150 mm × 4.6 mm, 4 μm)	75–2000 μg kg^{-1}	50–90 μg kg^{-1}	60–99%	[20]
HPLC–UV/vis	Sudan I–IV, Para Red	Red chilli pepper	Liquid extraction	9 min	ACE C18 (250 mm × 4.6 mm, 5 μm)	10–5000 μg L^{-1}	1.2–5.4 μg kg^{-1}	89–98%	[25]
HPLC–UV	Sudan I–IV, Para Red	Chilli spices	Ultrasound-assisted LLE	4 min	FastGradient Chromolith (50 mm × 2.0 mm)	100–5000 μg L^{-1}	35–100 μg L^{-1} for ST 500–2000 μg $kg^{-1}$for real samples	92–109%	[1]
HPLC–DAD	Sudan I–IV, Para Red	Chilli products	Ionic liquids extraction	25 min	Agilent Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm)	20–20,000 μg L^{-1}	7.0–13.2 μg L^{-1}	70.7–109.5%	[24]
HPLC–UV/vis	Sudan I–IV,Sudan Orange G, Fat Brown RR, M. Green C. B, Dimethyl Yellow, Solvent Blue 35, Sudan Black	Not applied for real sample	–	24 min	Symmetry C18 (150 mm × 3.0 mm, 3.5 μm)	400–5000 μg L^{-1}	60–146 μg L^{-1}	–	[22]
UPLC–DAD	Sudan I–IV, Para Red, Canthaxanthin, Astaxanthin	Feedstuff	SPE	6 min	Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm)	100–1000 μg L^{-1}	6–20 μg kg^{-1}	62.7–91.0%	[26]
UHPLC–UV/vis	Sudan I–IV, Sudan Red 7B, Sudan Red G, Sudan Orange G Para Red, Methyl Red	Chilli-containing spices	On-line SPE	9 min	Ascentis Express RP-Amide (100 mm × 4.6 mm, 2.7 μm)	20–10,000 μg L^{-1}	3.3–10.3 μg L^{-1} for ST 5.6–235.6 μg $kg^{-1}$for real samples	90–108%	The presented work

The results of LOD and LOQ for nine analytes in standard solutions and spiked real samples are listed in Table 2 and Table 3 respectively, which showed that the method met the requirements of the European Regulation that established the detection limit for Sudan's colorants in food matrices below 0.5–1 $\mu g\, g^{-1}$ or 500–1000 $\mu g\, kg^{-1}$ [3]. The limits of detection were in the range of 3.3–10.3 $\mu g\, L^{-1}$ for standard solutions, 5.6–143.5 $\mu g\, kg^{-1}$ for Chilli sauce, 6.1–73.2 $\mu g\, kg^{-1}$ for Feferony chilli-containing sauce and 8.7–235.6 $\mu g\, kg^{-1}$ for Mojo chilli-containing spices which demonstrated that the method is sufficiently sensitive.

As shown in Table 5, compared with earlier published results, limits of detection and quantification in proposed method are improved by using on-line SPE cleanup procedure. Previous reports described the LOD of standard solutions in the range of 35–100 $\mu g\, L^{-1}$ [1] for Sudan I–IV and Para Red (separation in 4 min), 50–90 $\mu g\, kg^{-1}$ [20] for Sudan I–IV. Obtained results in the present work can be compared to the method described by Qi [2] with LOD in the range of 4.1–5.8 $\mu g\, kg^{-1}$ for Sudan I–IV who used off-line SPE, but with longer time of separation (25 min for four compounds) and higher solvent consumption related to off-line SPE procedure. Proposed method is more advantageous than HPLC–UV/vis method described by Noguerol-Cal [22] who analyzed 10 colorants, including Sudan I–IV and Sudan Orange G, with LOD in range of 60–192 $\mu g\, L^{-1}$ (separation in 24 min). Moreover, the sensitivity of our method can be compared with HPLC–ESI-MS/MS method proposed by the same authors (LOD for standard solutions is in the range of 4.3–29.6 $\mu g\, L^{-1}$).

4. Conclusion

The new validated on-line SPE–UHPLC–UV/vis method using fused core particle columns provided fast and highly efficient chromatographic separation at low pressure (up to 20 MPa). This method proved to have a clear identification and accurate determination of nine of the most frequently found illegal dyes in complex matrices without the need of labor-consuming pretreatment in the real sample analysis. Due to the column switching system, method allowed an automated sample preparation step on a short fused core extraction precolumn, and showed advantages compared to a conventional off-line SPE method performed manually. Developed method provided faster analysis (in time less than 9 min including on-line SPE step) with higher sensitivity than earlier described methods with off-line SPE [2]. The advantages of the proposed on-line SPE–UHPLC method were the determination of a larger number of illegal colorants in a shorter time than previously described methods, with better values of recovery, precision, detection and quantification limits [23,11,24]. Even obtained sensitivity of the method can be compared with earlier described HPLC–MS method [22]. Based on the above results the present method can be recommended for the routine control of illegal dyes in chilli-containing samples. Moreover, the method described herein is the first procedure allowing the simultaneous extraction, and separation carried out using fused-core columns in both dimensions of UHPLC column switching system.

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