

## Simple determination of Sudan dyes in fruit juice and spices by microfunnel-filter-based emulsification microextraction followed by high performance liquid chromatography

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### Abstract

In this work, a rapid method termed as microfunnel-filter-based emulsification microextraction is coupled with high performance liquid chromatography for a simple determination of the banned Sudan dyes (I, II, and III) in fruit juice, spice, and water samples. According to the method, in order to speed-up the extraction and concentration of the target analytes, a micro-volume of a low-density organic solvent (1-octanol) is dispersed into an aqueous sample solution (45 mL), and a simple separation of the extractant phase is obtained using a syringe filter and microfunnel. The method provides a low-toxic extraction, it is centrifuge-less, and the extraction process is totally performed in a few minutes. The influencing factors including the type of organic solvent, number of extraction cycles, pH of the sample solution, and volume of the extraction solvent are investigated to achieve the optimal conditions. A good linearity (in the range of 0.7–1000 ng mL<sup>-1</sup> ( $R^2 > 0.995$ )) and a low limit of detection (in the range of 0.2–0.5 ng mL<sup>-1</sup>) are obtained, the extraction recovery is in the range of 94–107%, and the developed method provides a high enrichment factor (in the range of 294–354).

**Keywords:** Sudan dyes; Microfunnel emulsification microextraction; Juice and spice samples; Food matrices; High performance liquid chromatography.

### 1. Introduction

Sudan dyes are synthetic lipophilic azo dyes that are used in many industrial products such as textile, cosmetics, wood, oils, plastics, and leather. Owing to the evident toxic effects of these compounds on the human organs, they have been classified as genotoxic carcinogens by the International Agency for Research Cancer (IARC) and there are clear laws in many

countries to ban the use of these dyes in foods [1]. Despite these considerations, due to the low price and the intense color of the Sudan dyes, these compounds are added illegally to various foodstuffs such as spices, tomato sauces, fruit juice, and many other frequently eaten foods. Moreover, their extensive use in the mentioned products is a threat to the environment, and they can efficiently pollute waters [2]. Hence,

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existence of reliable, accurate, and simple methods for the determination of these dyes in the food samples is a necessity [3], and extensive investigations are ongoing for this purpose. Different methods including high performance liquid chromatography (HPLC) [1, 4], surface-enhanced Raman scattering spectroscopy (SERS) [5], and voltammetry [6] have been reported for determination of the Sudan dyes in various samples. Sample preparation is an integral and essential step in most methods used for determination of these dyes, and is utilized to increase the compatibility of samples with analyzer systems, sample clean-up, and concentration of the analytes before the final analysis to increase the method sensitivity. The microextraction techniques are new versions of sample preparation methods that have been introduced to address some drawbacks of the conventional extraction methods such as a high consumption of large amounts of highly expensive and potentially hazardous organic solvents and time-consumption. Microextraction based on dispersion of the extractant into the sample solution is a simple procedure that can provide a high enrichment factor for the analytes in a short period of time. Dispersive liquid-liquid microextraction (DLLME) is a distinguished method in this category, and since its introduction, it has immediately gained considerable attention by the researchers [7-11]. The valuable capabilities of this method have caused a new version of this method to be introduced to modify its deficiencies including the consumption of highly toxic solvents such as the halogenated ones, providing a low sample clean-up, utilization of the disperser solvent, and using a centrifugation step [12-15].

Our research team has recently introduced a simple method termed as microfunnel-filter-based emulsification microextraction to address these drawbacks [16]. The method is centrifugeless and disperser-free, provides a suitable sample clean-up, and by utilization of a low-density organic solvent, it can exhibit an environmentally friendly sample treatment. In this work, for the first time, microfunnel-filter-based emulsification microextraction coupled with HPLC

was used for a simple determination of the banned Sudan dyes (I, II, and III) in fruit juices, spices, and water samples. According to this method, a micro-volume of a low-density organic solvent is dispersed into a high volume of an aqueous sample solution to speed-up the extraction, and a simple separation of the extractant phase is obtained using a syringe filter and a microfunnel. Also the method can provide a high enrichment factor in a few minutes.

## 2. Materials and methods

### 2.1. Chemicals and supplies

1-octanol ( $\geq 95.0\%$ ) was supplied from Sigma-Aldrich (St. Louis, USA) and dihexyl ether (97.0%) was obtained from Fluka (Buchs, Switzerland). Pure water was obtained by a Milli-Q water purification system (Millipore, USA). NaOH ( $\geq 99.0\%$ ), hydrochloric acid (37%),  $H_3PO_4$  (85%), analytical-grade sodium chloride ( $\geq 99.0\%$ ), chromatography-grade methanol, acetonitrile, and acetone were obtained from Merck (Darmstadt, Germany). Sudan I, Sudan II, and Sudan III were supplied from Sigma-Aldrich Corporation (St. Louis, MO, USA); stock solutions of these dyes with a concentration of  $1 \text{ mg mL}^{-1}$  were prepared in methanol and stored at  $4^\circ\text{C}$  in a refrigerator and re-prepared every 3 weeks. The other chemicals used were of analytical reagent grade.

### 2.2. Preparation of samples

The chili sauce and fruit juice samples were collected from a local market. The fruit juice samples were filtered through a 0.45 mm syringe filter, and 10 mL of the passed phase was diluted by pure water (pH 13.0) to 45 mL and directly extracted by the proposed method. A certain amount of the chili sauce samples (0.5 g) was accurately weighed, and then using an ultrasonic water bath, the target analytes were extracted into 4.5 mL of methanol at  $25^\circ\text{C}$  in 30 min. The extractant phase was filtrated by a 0.45 mm syringe filter, and finally, the passed phase was diluted to 45 mL with pure water (pH 13.0) and extracted by an optimized extraction protocol. The wastewater samples were collected in amber glass containers and maintained in the dark at  $4^\circ\text{C}$  until analysis; they were

filtered through a 0.45 mm syringe filter, and the proposed method was directly applied on them.

### 2.3. Instrumentation

The absorbance spectra of the sample solutions were obtained by a UV-visible detector (Jasco, Tokyo, Japan). The pH values of the solutions were monitored by a Metrohm pH-meter, model 654 (Herisau, Switzerland). A Knauer HPLC instrument (Berlin, Germany) equipped with a D-14163 degasser, a pump of K-1001 HPLC, and a K-2600 UV detector was utilized for the chromatographic separation and analysis of the dyes. An ODS III (5  $\mu\text{m}$  particle diameter, 250 mm  $\times$  4.6 mm i.d.) supplied from MZ Analysen Technik (Mainz, Germany) was utilized for separation of the target analytes. A mixture of methanol, acetonitrile, and 0.5% (v/v) aqueous acetic acid solution (85:10:5, v/v/v %) under an isocratic elution and a flow rate of 1 mL  $\text{min}^{-1}$  was used as the mobile phase. A wavelength of 480 nm was utilized for detection of all the target analytes. Syringe filters with nylon membranes (0.45  $\mu\text{m}$ , Millex-HN, nylon, Ireland) were used for phase separation, and a home-made microfunnel was used for collection of the extractant phase.

### 2.4. Procedure

Microfunnel-filter-based emulsification microextraction is a two-phase method, in which a micro-volume of an organic solvent (1-octanol, 150  $\mu\text{L}$ ) is dispersed into a high volume of an aqueous sample solution (45 mL, pH 13.0) to speed-up the extraction process. According to the method, after addition of the organic solvent to the sample solution, the mixture was repeatedly sucked into a glass syringe and dispensed into the sample container to disperse the extractant phase into the sample solution. Each dispensing and suction cycle caused the mixture to become more and more turbid, and the solution became totally cloudy by repeating 15 times of this process. Then the mixture was passed through a syringe filter with a nylon membrane for phase separation. A nylon membranes is permeable to a broad range of solvents. Hence, along with passing the cloudy mixture through

it, the dispersed organic solvent was aggregated, separated from the aqueous sample solution, and floated on the aqueous phase. In order to collect the organic phase, a home-made microfunnel was used, and by adding a few milliliters of ultrapure water to the vial through the rubber cap, the extractant phase was raised and narrowed into the capillary part of the microfunnel. Finally, the organic phase was collected using a micro-syringe and transferred to HPLC for final analysis.

### 3. Results and discussion

In order to obtain the best results, the parameters affecting the efficiency and speed of the extraction method should be investigated and optimized. Based on the method, increasing the surface area between the donor and acceptor phases to speed-up the process is achieved by dispersion of the organic solvent into the donor phase. Hence, the extraction number is a parameter that can affect the mass transfer rate. Also there are some parameters including the type of organic solvent, sample solution pH, and volume of the extraction solvent, which can be influential on the extraction recovery and should be optimized.

**Equations 1** and **2** are used to calculate the enrichment factor (EF) for the target analytes and the percent extraction recovery (ER%) for the method, respectively.

$$EF = \frac{C_{a,final}}{C_{s,initial}} \quad (1)$$

$$ER\% = \frac{n_{a,final}}{n_{s,initial}} \times 100\% = EF \times \frac{V_a}{V_s} \times 100\%$$

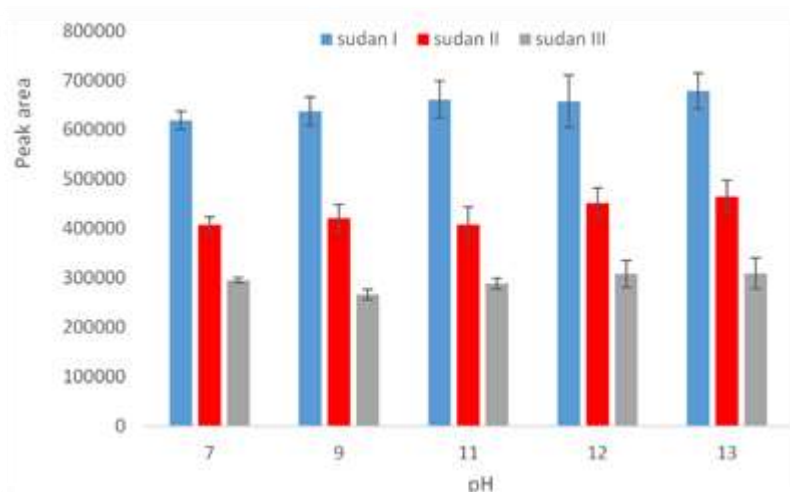
(2)

where  $n_{s,initial}$  and  $n_{a,final}$  are the number of moles of the analyte originally present in the sample solution and the number of moles of the analyte finally collected from the acceptor solution; the volume of the sample solution is specified as  $V_s$ , and  $V_a$  is the volume of the acceptor solution;  $C_{s,initial}$  is the initial analyte concentration in the sample solution, and the final concentration of the analyte present in the extractant phase is specified as  $C_{a,final}$ .

### 3.1. pH of sample solution

Extraction of the understudied Sudan dyes from aqueous sample solutions can be dependent on the pH of the solutions. Hence, the effect of the sample solution pH on the extraction efficiency was investigated in the pH range of 7.0-13.0. The results

obtained are shown in **Fig. 1**. As it can be seen, the extraction efficiency was slightly enhanced by increasing the sample solution pH up to 12.0, and then it remained nearly constant. Based on the results obtained, pH 13.0 was chosen for further experiments.

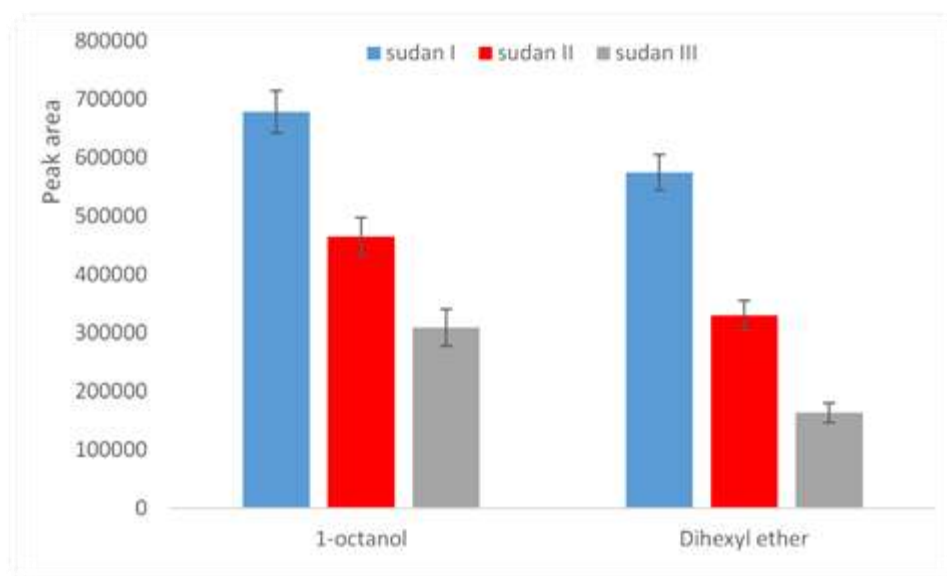


**Fig 1:** Effect of sample solution pH on the extraction efficiency. Conditions: sample solution, 45.0 mL of 100 ng mL<sup>-1</sup> of each analyte in deionized water; extraction solvent, 1-octanol; volume of organic solvent, 500  $\mu$ L; extraction number, twenty times. The error bars were obtained based on three replicates.

### 3.2. Type of organic solvent

In a two-phase extraction method, the type of organic solvent can efficiently affect the distribution coefficient of the analytes present between the phases and the method efficiency. In addition to have a suitable chemical nature to provide an efficient extraction of the target analytes from the sample solution, the organic solvent should have some properties including a low solubility in the sample solution and having a sufficient boiling point to prevent the evaporation of the solvent during the extraction. Also toxicity of the extraction process is effectively dependent on the type of organic solvent, and in order

to follow the green chemistry concerns, it should have a low toxicity as much as possible. Organic solvents with a density lower than water can generally provide low-toxic extractions compared to the solvents with a density higher than water, especially the halogenated ones. Hence, in this work, two low-density organic extraction solvents including 1-octanol and dihexyl ether were investigated as the extraction solvent, and the results obtained were shown in **Fig. 2**. As it could be seen, both extraction solvents could introduce suitable extractions but 1-octanol provided better results, and thus it was chosen as the extraction solvent.



**Fig 2:** Effect of the type of organic solvent on the extraction efficiency. Conditions: sample solution, 45.0 mL of 100 ng mL<sup>-1</sup> of each analyte in deionized water (pH, 13.0); volume of organic solvent, 300  $\mu$ L; extraction number, twenty times. The error bars were obtained based on three replicates.

### 3.3. Number of extraction cycles

As mentioned earlier, microfunnel-filter-based emulsification microextraction is a very fast method, in which dispersing the organic solvent into the sample solution causes to increase the surface area between the phases and enhances the mass transfer rate. Unlike the conventional dispersive-based methods that use a chemical disperser, in the proposed method, dispersion of the acceptor phase into the sample solution is achieved by utilization of a glass syringe. For this purpose, after addition of the organic solvent to the sample solution, the mixture was repeatedly sucked

### 3.4. Volume of organic solvent

The volume of the organic solvent plays a key role in the proposed method. This parameter can efficiently affect the extraction recovery of the method and the enrichment factor for the target analytes. It is obvious that in a two-phase extraction method, increasing the volume of the extraction solvent can lead to enhance the extraction recovery for all analytes. On the other hand, it must be noted that the effect of the volume of the extraction solvent on the enrichment factor is not direct, and by an excess increase in the volume of the organic solvent, the enrichment factor can be decreased by diluting the acceptor phase. By considering these

into a syringe and dispensed into the sample container; this is referred to as the number of extraction cycles. The dispensing and suction cycles caused the mixture to become more and more turbid, and in order to optimize the extraction number, this parameter was investigated in the range of 5-20. The results obtained show that by increasing the extraction number in the range of 5-15, the extraction efficiency was improved as a result of the enhancement of the mass transfer rate, and with a further increase in the extraction cycles, it was almost unchanged. Hence, fifteen extraction cycles were selected for further studies.

criteria to examine the effect of the volume of the extraction solvent, the solutions containing various volumes of 1-octanol, in the range of 150-500  $\mu$ L, were evaluated by the same procedure. The results obtained are shown in **Fig 3**. Based on these results, the analytical signals for the target analytes were decreased as a result of increasing the volume of the extraction solvent, and 150  $\mu$ L of this solvent provided the best enrichment factor. Therefore, 150  $\mu$ L was chosen as the optimized volume for the extraction solvent. Also the extractant phase is 75  $\mu$ L.

Also to evaluate the effect of the ionic strength of the sample solution on the extraction efficiency, different

amounts of NaCl (0–25%, m/v) were added to the sample solution, and the results obtained showed that

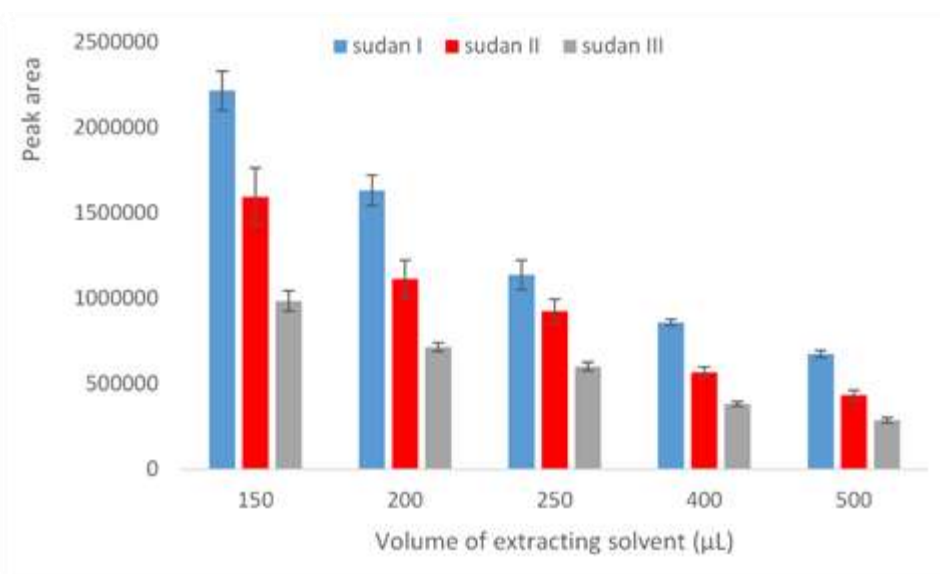
### 3.5. Evaluation of method

The figures of merit of the developed microfunnel-filter-based emulsification microextraction method for extraction of the understudied dyes were evaluated by investigating some quantitative characteristics including the percent extraction recovery (ER%), enrichment factor (EF), repeatability, linearity, limit of detection (LOD), and limit of quantification (LOQ).

For this purpose, the linearity of the method was studied using the deionized water samples spiked with the target dyes at nine different concentrations ranging from 0.7 to 1000 ng mL<sup>-1</sup>, and the calibration curves obtained exhibited the coefficient of determination ( $r^2$ ) > 0.995 (Table 1). The experimental method was used to evaluate LOD and LOQ of the method for each analyte. On this basis, LODs were obtained based on a

the best signals could be obtained in 10% (m/v) of the salt.

signal-to-noise ratio of 3; they were in the range of 0.2–0.5 ng mL<sup>-1</sup>. Also the method provided LOQs in the range of 0.7–1.5 ng mL<sup>-1</sup> based on a signal-to-noise ratio of 10. The peak areas for the target analytes were studied for five replicate extractions, and deionized water was spiked at the 100 ng mL<sup>-1</sup> level to evaluate the repeatability of the method. The relative standard deviation (RSD) was used to express the precision of the method; it was satisfactory, ranging from 5.9% to 8.3% for all the target analytes. Equations 1 and 2 were utilized to calculate ER% and EF of the method for the understudied dyes. The results obtained showed that the absolute extraction values were between 49% and 59%, and the EF values were in the range of 294–354.



**Fig 3:** Effect of the volume of the organic solvent on the extraction efficiency. Conditions: sample solution, 45.0 mL of 100 ng mL<sup>-1</sup> of each analyte in deionized water (pH, 13.0); extraction solvent, 1-octanol; extraction number, fifteen times. The error bars were obtained based on three replicates.

### 3.6. Analysis of real samples

The applicability of the method to determine the target Sudan dyes in some real samples including fruit juice, wastewater, and spices was studied, and the results obtained were shown in Table 2. These samples were collected and initially prepared based on the procedures provided in Section 2.2. All samples were initially analyzed by the proposed method to determine

the concentration of the target dyes, and then in order to investigate the matrix effects, they were spiked with proper amounts of the analytes, and the percent relative recoveries (RR%, Equation 3) were subsequently calculated.

$$RR\% = \frac{C_{found} - C_{initial}}{C_{add}} \times 100 \quad (3)$$

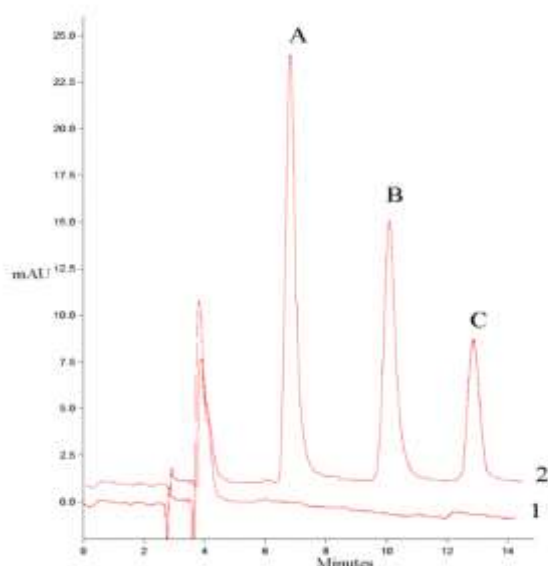
where  $C_{found}$  and  $C_{initial}$  are the concentrations of the analytes after the extraction procedure and in the initial

sample solution, respectively, and  $C_{add}$  is the added concentration of the analytes. The different matrices used for the fruit juice and wastewater samples had no significant effect on the extraction efficiency, and to determine the target analytes in the sample solutions, the method was directly utilized. On the other hand, the results obtained revealed that the matrix of the chili sauce sample had a significant effect on the method.

Hence, the standard addition method was used to determine the target analytes in these sample solutions. As it could be seen in **Table 2**, the percent relative recoveries for all the real samples ranged from 94% to 107%, and the chromatograms related to the fruit juice samples for the non-spiked and spiked analytes at the concentration level of  $25 \text{ ng mL}^{-1}$  were shown in **Fig. 4**.

**Table 1.** Figures of merit for the proposed method for the target analytes.

Element	LOD <sup>a</sup>	LDR <sup>b</sup>	R <sup>2c</sup>	RSD% <sup>d</sup> (n = 5)		ER% <sup>e</sup>	PF <sup>f</sup>
				Intra-day	Inter-day		
Sudan I	0.2	0.7-1000	0.998	7.8	(7.8)	49	294
Sudan II	0.3	1.0-1000	0.995	5.9	(8.1)	53	318
Sudan III	0.5	1.5-1000	0.996	6.6	(8.3)	59	354



**Fig 4:** HPLC-UV chromatograms for (1) non-spiked and (2)  $25.0 \text{ ng mL}^{-1}$  of each sudan dye-spiked juice fruit sample (A) Sudan I, (B) Sudan II, (C) Sudan III.

#### 4. Concluding remark

A fast method termed as microfunnel-filter-based emulsification microextraction was coupled with HPLC for a simple determination of the banned Sudan dyes (I, II, and III) in the foodstuff samples. There are a number of methods available for the extraction of these compounds. Some of these methods were compared with microfunnel-filter-based emulsification microextraction method (Table 3) [17-19]. The method is very fast so that it can be implemented in a few minutes, it is centrifuge- and disperser-free, and by utilization of a micro-volume of a low-toxic extraction solvent, it provides an environmentally friendly procedure.

<sup>a</sup>Limit of detection (S/N = 3), ( $\text{ng mL}^{-1}$ ).

<sup>b</sup>Linear dynamic range ( $\text{ng mL}^{-1}$ ).

<sup>c</sup>Coefficient of determination.

<sup>d</sup>Relative standard deviation (n = 5, C =  $100 \text{ ng mL}^{-1}$ ).

<sup>e</sup>Percent extraction recovery (C =  $25 \text{ ng mL}^{-1}$ ).

<sup>f</sup>Preconcentration factor.

**Table 2.** Analysis of real samples under the optimal experimental conditions.

Sample		Sudan I	Sudan II	Sudan III
Fruit juice	Added <sup>a</sup>	50.0	50.0	50.0
	Found <sup>b</sup>	47.1	51.6	49.7
	RR% <sup>c</sup>	94.2	103.2	99.4
	RSD% (n = 3)	8.3	7.1	6.6
Chili sauce	Added <sup>d</sup>	10.0	10.0	10.0
	Found <sup>e</sup>	10.2	9.8	9.6
	RR%	102.0	98.0	96.0
	RSD% (n = 3)	6.9	5.2	7.3
Wastewater	Added	25.0	25.0	25.0
	Found	23.4	24.1	26.8
	RR%	93.6	96.4	107.2
	RSD% (n = 3)	6.2	5.0	4.1

**Table 3.** Comparison between proposed method and other extraction methods for determination of Sudan dyes.

Analytical method <sup>f</sup>	Analyte	LOD (ng mL <sup>-1</sup> )	LDR (ng mL <sup>-1</sup> )	ER%	PF	Analysis time (min)
AALLME/ HPLC-UV	Sudan I	7.7	27-2900	-	37	4.5
	Sudan II	8.3	29-2300	-	34	
	Sudan III	12.1	40-2600	-	37	
Ultrasound-assisted LLE/HPLC-DAD	Sudan I	50	200-5000	-	-	20
	Sudan II	60	200-5000	-	-	
	Sudan III	70	200-5000	-	-	
U-HF-LPME-HPLC-UV	Sudan I	0.09	1.0-1000	-	186	≥40
	Sudan II	0.31	1.0-1000	-	52	
	Sudan III	0.63	5.0-1000	-	38	
ILE-HPLC/UV	Sudan I	12.8	20-20000	-	-	32
	Sudan II	12.0	20-20000	-	-	
	Sudan III	11.2	20-20000	-	-	
This work	Sudan I	0.2	0.7-1000	49	294	10.0
	Sudan II	0.3	1.0-1000	53	318	
	Sudan III	0.5	1.5-1000	59	354	

In order to obtain the best results, the effective parameters involved were optimized, and the method introduced a high EF in the range of 294-354 for the analytes and a suitable extraction repeatability in the optimal conditions. The EF values obtained caused the method to provide low limits of detection and quantification for the target analytes, and providing a

#### Acknowledgment

wide linear dynamic range was another capability of the method. The method was successfully applied for determination of the target analytes in the fruit juice, wastewater, and chili sauce samples, and the RR% values for the method were obtained to be in the range of 94-107.

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<sup>a</sup>Spiked concentration (ng mL<sup>-1</sup>).

<sup>b</sup>Concentration of analytes (ng mL<sup>-1</sup>) in sample after spiking target analytes.

<sup>c</sup>Relative recovery.

<sup>d</sup> Spiked concentration (ng mg<sup>-1</sup>)

<sup>e</sup> Concentration of analytes (ng mg<sup>-1</sup>) in sample after spiking target analytes

<sup>f</sup> Air-assisted liquid-liquid microextraction (OS-AALLME), High performance liquid chromatography (HPLC), U-shaped-Hollow fiber-liquid phase microextraction (U-HF-LPME), Dual solvent-stir bars microextraction (DSSBME), Ionic liquid-based extraction (ILE), Ultrasound-assisted emulsification microextraction (USAEME).



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