Comparison of Two Liquid-Liquid Microextractions for the Detection of Crocin in the Saffron and Biological Samples Using UV- Vis Spectrophotometry

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ABSTRACT: *In this work, two approaches based on Dispersive Liquid-Liquid MicroExtraction (DLLME) and Dispersive Liquid-Liquid MicroExtraction based on Solidification of Floating Organic Drop (DLLME-SFO) were compared for the extraction and preconcentration of crocin from saffron and biological samples. Different DLLME and DLLME-SFO parameters influencing the extraction efficiency were studied and optimized. The results showed that both extraction methods exhibited good linearity, precision, enrichment factor, and detection limit. Under optimal conditions, the limits of detection were 0.008 and 0.005 ng/mL for DLLME and DLLME-SFO, respectively. The preconcentration factors were 88 and 95 for DLLME and DLLME-SFO, respectively. The applicability of the proposed methods was examined by analyzing crocin in saffron, urine and milk samples and good results were obtained. The percentage recovery values for spiked samples were between 96.4 and 99.1.*

KEYWORDS: *Dispersive liquid-liquid microextraction; Dispersive liquid-liquid microextraction based on solidification of floating organic drop; Crocin; Saffron; Biological samples.*

INTRODUCTION

Crocin (digentiobiosyl 8, 8'-diapocarotene-8, 8'-oate; $C_{44}H_{64}O_{24}$) belongs to a group of natural carotenoid obtained commercially from the dried trifid stigma of the culinary spice *Crocus sativus* L. It is the diester formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin. It had a deep red color and formed crystals with a melting point of 186°C. One of the few naturally occurring carotenoids easily soluble in water. Crocin is the chemical ingredient primarily responsible for the color of saffron. Structure of Crocin was elucidated by Karrer [1] though its presence was reported by *Aschoff* as long ago as the 19th century, is the main pigment in the saffron (approx. 80%). With water as a stationary phase and butanol as mobile phase, Crocin can be isolated in pure form from the saffron extract and directly crystallized.

Crocin scavenges free radicals, especially superoxide anions, and so may protect cells from oxidative stress. Crocin is useful as sperm cryopreservation and in protecting hepatocytes from toxins. Because of its powerful antioxidant activity, it could be useful in the therapy of neurodegenerative disorders [2-3].

UV- Vis spectroscopy has been widely used for

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the determination of crocin in saffron samples because it is a relatively simple technique and inexpensive equipment. However, the direct determination of crocin by UV- Vis spectroscopy is limited not only by insufficient sensitivity but also by matrix interference especially in biological samples, therefore, suitable extraction methods are required for determining low concentrations of crocin in saffron and biological samples.

Liquid–Liquid Extraction (LLE) [4] and Solid-Phase Extraction (SPE) [5-6] are important techniques for the extraction of analytes from liquid samples. Recently several different types of Liquid-Phase Micro Extraction (LPME) methods have been developed, including Dispersive Liquid-Liquid MicroExtraction (DLLME) [7] and Dispersive Liquid-Liquid MicroExtraction based on Solidification of Floating Organic drop (DLLME-SFO) [8].

DLLME involves the use of a mixture of two solvents (extraction and dispersive), which is injected into the aqueous sample forming a cloudy solution. The high dispersion of extraction solvent accelerates the analyte extraction and after a centrifugation step is possible to collect the organic phase (extract) [9-11]. The main characteristics of DLLME are simplicity, low cost, suitable analyte recovery, high preconcentration factors, low solvent consumption and short time for extraction [12].

DLLME-SFO use solvents with the densities lower than water and lower toxicity as extractant solvent. Since the melting point of the extraction solvent is low (in the range of 10–30 °C); the organic drop could be solidified at low temperatures, so it was easily scoped out from the water sample. The vast contact area between the extraction solvent and the sample resulted in a faster mass transfer and shorter extraction time [13]. The advantages of DLLME-SFO are simplicity, high efficiency, rapidity, high recovery and higher extraction efficiency for heavy metal ions. Other advantages are low cost, simple extraction apparatus and consumption of very small amounts of less-toxic organic solvents. In addition, the extraction time is even shorter than Solidified Floating Organic Drop MicroExtraction (SFODME).

In this method, there is no need to use a conical bottom glass tubes, which are easily damaged and difficulty cleaned. The floated extractant is solidified and easily collected for analysis.

To the best of our knowledge, there is no publication related to the comparison between the efficiencies of two types of LPMEs (DLLME and DLLME-SFO). The goal of this study is to compare the suitability of DLLME and DLLME-SFO for the preconcentration and determination of trace amounts of crocin in saffron and biological samples. Several factors affecting the extraction efficiencies of the methods were scrutinized. Finally, the developed methods were validated through the analysis of crocin in saffron, urine and milk samples.

EXPERIMENTAL SECTION

Chemicals and reagents

All reagents were of analytical reagent grade and deionized water was used throughout. Stock solution (1000 mg/L) of crocin was prepared by direct dissolution of proper amounts of crocin from Merck (Darmstadt, Germany) in deionized water and stored in the dark at 4˚C and was diluted with deionized water to obtain a working standard solution with a concentration of 0.1 µg/mL. Other reagents including 1-undecanol, carbon tetrachloride, dichloroethane, dichloromethane, chloroform, Ortho-xylene, acetone, ethanol, and acetonitrile were purchased from Merck. The pH of solutions was adjusted by NaOH (0.5 mol/L) and dropwise addition of HCl (0.5 mol/L). Sodium chloride (Merck) was of the highest purity available.

Apparatus

Absorbance measurements were carried out on a UV–Vis spectrophotometer model JENWAY 6300 using quartz microcell in the wavelength ranges 320-1000 nm for recording absorption of crocin. Tungsten halogen lamp was used as the radiation source with the Spectral bandwidth 8 nm and Wavelength resolution 1 nm. A digital pH meter Metrohm, Model NANO Technic was used for all pH measurements. A Denley bench centrifuge model BS400 (Denley Instruments Ltd., Billingshurst, UK) was used to accelerate the phase separation. A Hamilton syringe was used for rapid injection.

Extraction procedure

Dispersive liquid-liquid microextraction

A 10 mL of a standard solution or real sample, the pH was adjusted to 9, was placed into 10 mL test tube, and a mixture of 200 µL dichloromethane as an extraction solvent and 400µL aceton as a dispersive

solvent was rapidly injected into the aqueous sample containing crocin using 1.0 mL syringe. A cloudy solution, resulting from the dispersion of fine dichloromethane droplets in the aqueous solution was formed in the test tube. In order to accelerate phase separation, the solution was centrifuged for 8 min at 4000 rpm. After this step, the dispersed fine droplets of dichloromethane were settled at the bottom of the tube. The aqueous phase was discarded with a syringe and the remaining organic phase was transported to a UV–Vis spectrophotometer using quartz microcell to measure its absorbance at λ_{max} (440 nm) for the determination of crocin.

Dispersive liquid-liquid microextraction based on solidification of floating organic drop

10 mL of crocin solution, the pH was adjusted to 9, was placed into 10 mL test tube, and a mixture of 200 µL 1-undecanol (extraction solvent) and 700 µL methanol (dispersive solvent) was rapidly injected into the sample solution. In this stage, a cloudy solution containing many dispersed fine 1-undecanol was formed and crocin was extracted into 1-undecanol in a few seconds. Then, the mixture was centrifuged for 6 min at 4000 rpm, the organic solvent droplet was floated on the surface of the aqueous solution due to its low density. The vial was then transferred into an ice bath and the organic solvent was solidified after 10 min and the solidified solvent was transferred into a conical vial where it melted immediately. After this process, the extract was collected and was transported to a UV–Vis spectrophotometer.

Preparation of real samples

Saffron samples obtained from the different area of Khorasan state of Iran contain 3 regions (Ttorbatheydariyeh, Ghaen, Bakharz) in the year 2015. Saffron samples milled to make a fine powder before spectrophotometry. 50 mg of saffron dissolved in 70 mL water slowly using a magnetic shaker for 1 hours and the final volume made to 100 mL. 10 mL of this sample taken and dilution makes to 100 mL. pH of the saffron sample was adjusted at pH 9 using concentrated NaOH; then 10 ml of sample was applied to DLLME and DLLME-SFO.

Milk and urine samples were pretreated by adding 3 mL methanol and 5 mL water to a 3 mL portion of the solutions and centrifuging it for 20 min. After filtration, the pH of the 5 mL solutions was then adjusted to pH 9.0 using NaOH and diluted to 50 mL. Then 10 ml of diluted sample was applied to DLLME and DLLME-SFO.

RESULTS AND DISCUSSION

In order to obtain the optimized extraction condition, Extraction Recovery (ER) was used to evaluate the optimum condition. ER% was defined as the percentage of the total analyte (n_0) extracted into the supernatant phase (n_{sup}). Accordingly, calculation of the extraction recovery, as an analytical response, was carried out using the following equation:

$$ER\% = n_{sup}/ n_0 = C_{sup} \times V_{sup} / C_0 \times V_{sam} \times 100 \quad (1)$$

Where C_{sup} and C_0 are the concentrations of analyte in the supernatant phase and initial concentration of an analyte in an aqueous sample, respectively. C_{sup} is determined from a calibration curve which was obtained using direct injection of standard solutions. V_{sup} and V_{sam} are the volumes of the supernatant phase and aqueous sample, respectively.

The Preconcentration Factor (PF) was defined as the ratio between the analyte concentration in the supernatant phase (C_{sup}) and the initial concentration of the analyte (C_0) in the aqueous sample, as follows:

$$PF = C_{sup} / C_0 \quad (2)$$

Combination of Eqs. (1) and (2) gives:

$$ER\% = PF \times V_{sup} / V_{sam} \times 100 \quad (3)$$

To obtain good sensitivity and precision for extraction and determination of crocin, the various experimental parameters which influence the efficiency of DLLME and DLLME-SFO procedures including extracting and disperser solvents, as well as their volume, pH of the solution, centrifugation time and salt addition, were optimized.

Extraction of crocin by DLLME

Optimization of DLLME

The type of dispersive and extraction solvents used in DLLME is an essential consideration for efficient extraction. The extraction solvent should be higher density than water, high extraction capability of the interesting compounds and low solubility in water and dispersive solvent should be miscible with both water and

Table 1: Effect of type of the disperser solvent and extraction solvent on DLLME extraction recovery of crocin (n=3).

//////////////////////////	Mthanol	Acetone	Acetonitrile	Ethanol
Chloroform	1.51±13.05	1.31±10.00	1.11±9.69	1.12±14.12
Dichloromethane	1.41±65.71	1.12±87.86	1.10±14.28	0.95±43.00
Ortho-xylene	0.60±5.65	0.95±2.86	0.98±10.71	0.98±6.22
Dichloroethane	1.01±37.91	1.51±14.29	1.22±21.93	1.11±20.92
Tetrachloridcarbon	1.60±51.81	0.52±61.00	2.10±20.09	1.98±49.12

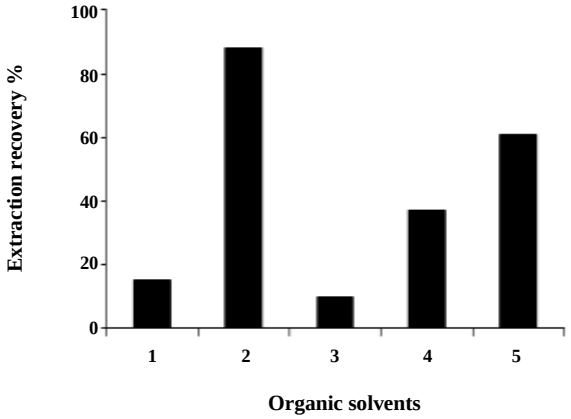

Fig. 1: Effect of type of extraction solvent on DLLME extraction recovery of crocin (Acetone disperser solvent). 1: Chloroform 2: Dichloromethane 3: Ortho-xylene 4: Dichloroethane 5: Tetrachloridcarbon

the extraction solvent [14-16]. Therefore, acetonitrile, acetone, ethanol, and methanol were tested as the dispersive solvents and chloroform, Dichloromethane, Ortho-xylene, Dichloroethane, and carbon tetrachloride were studied as the extraction solvents in the extraction of crocin. For obtaining good efficiency, all combinations using chloroform, Dichloromethane, Ortho-xylene, Dichloroethane, and carbon tetrachloride (400 µL) as extractant with acetone, acetonitrile, ethanol, and methanol (1 mL) as a dispersive solvent were tried. As results shown in Fig. 1 and Table 1 indicate, acetone as the disperser solvent and Dichloromethane as the extracting solvent provided maximum extraction recovery of 87.86%. Therefore, we selected aceton/ dichloromethane as a suitable set for the subsequent experiment.

Solvent volume is a key parameter affecting the extraction kinetics and also the preconcentration factor. To consider the effect of disperser solvent volume on extraction recovery, different volumes of aceton were tested. Therefore, the volume of the extracting solvent (dichloromethane) was fixed at 200 µL and the volume of aceton was changed from 200 to 1000 µL. The represented results in Fig. 2a, shows that with increasing the volume of aceton, extraction recovery first increased till reached a maximum point of 400 µL and then gradually decreased by further increasing of its volume. It can be attributed to the fact that, at a lower volume of aceton consumption, cloudy state was not formed well and the extracting solvent (Dichloromethane) could not be well dispersed among aqueous solution in the form of very little droplet, which resulted in poor extraction recovery. Therefore, in the following experiments, 400 µL aceton was used as optimal disperser solvent volume.

In all extraction techniques extracting solvent volume is one of the essential factors which influence the extraction efficiency. The rate of the analyte transport into the solvent microdrop is directly related to the interfacial area between the aqueous phase and extracting phase and inversely related to the extracting phase volume [17]. So, by increasing the drop volume, interfacial area and also extraction efficiency is enhanced. By further increase in the drop volume, the effect of the solvent volume is predominated and extraction efficiency is decreased. To consider the effect of the extracting solvent volume on extraction recovery, different volumes of Dichloromethane were tested. Therefore, the volume of disperser solvent (aceton) was fixed at 400 µL and the volume of Dichloromethane was changed from 100 to 500 µL. According to Fig 2b, it is clear that the optimum level of crocin was extracted when the volume of Dichloromethane was 200 µL. Hence 200 µL of Dichloromethane was selected for the subsequent experimental work.

pH of the sample is an important factor during Liquid-Liquid Extraction (LLE) process involving analytes that possess an acidic or basic moiety [18]. The ionic form of a neutral molecule formed upon deprotonation of a weak acid or protonation of a weak base normally

does not extract through the organic solvent as strongly as its neutral form does. Thus pH should be adjusted to ensure that neutral molecular forms of the analytes are present prior to performing the microextraction step. In this step, the effect of pH of the solution on the amount of extracted crocin was investigated in the range of 3–12. As can be seen in Fig. 3, the best pH for extraction of crocin is 9, that crocin is completely in its molecular form.

The influence of ionic strength on the efficiency of microextraction of the proposed (DLLME) procedure was investigated by adding different concentrations of NaCl in the range of 0–10% (w/v). According to the experimental results obtained, the salt addition has no significant effect on the extraction efficiency of crocin. Therefore, all the extraction experiments were carried out without the addition of salt.

The effect of centrifugation time upon extraction efficiency was studied in the range of 2–10 min. As shown in Fig. 4, a centrifugation time of 8 min was selected as optimum; since complete phase separation occurred at the end of this period, while at lower or higher centrifuge time, the recoveries were both lower.

Extraction of crocin by DLLME-SFO

Optimization of DLLME-SFO

In order to obtain high recovery, the selection of extraction solvent has an important role in the DLLME-SFO system. Extraction solvent should have special characteristics; it should have lower density rather than water, high efficiency in the extraction of the interesting compounds and low solubility in water and it should have a melting point near room temperature was floated on the surface of the aqueous solution. According to these considerations, 1-undecanol was chosen as the extracting solvent [19- 21].

Miscibility of a disperser with organic phase (extraction solvent) and aqueous phase (sample solution) is the most important point for the selection of a disperser. Therefore, acetone, acetonitrile, methanol, and ethanol, which have this ability, are selected for this purpose. For obtaining maximum extraction recovery, all combinations using 1-undecanol as extractant with acetone, acetonitrile, methanol, and ethanol as dispersive solvent, were examined. According to the results shown in Table 2, methanol as the disperser solvent provided maximum extraction recovery.

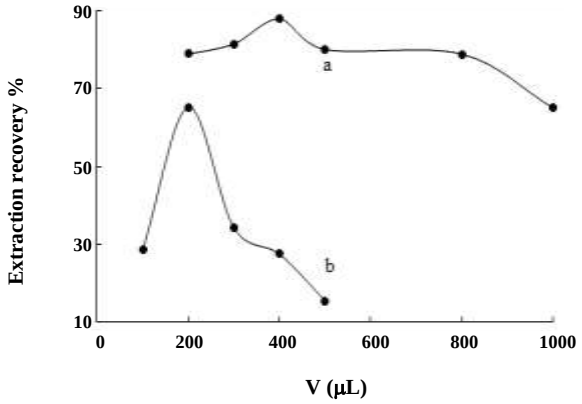

Fig. 2: (a) Effect of volume of disperser solvent, aceton on DLLME efficiency. Extraction conditions: volume of the aqueous solution, 10 mL (containing 0.1 µg/mL of crocin); and volume of Dichloromethane, 200 µL. (b) Effect of volume of extraction solvent, dichloromethane on DLLME efficiency. Extraction conditions: as in Fig. 2a, except that volume of disperser solvent, aceton is 400 µL.

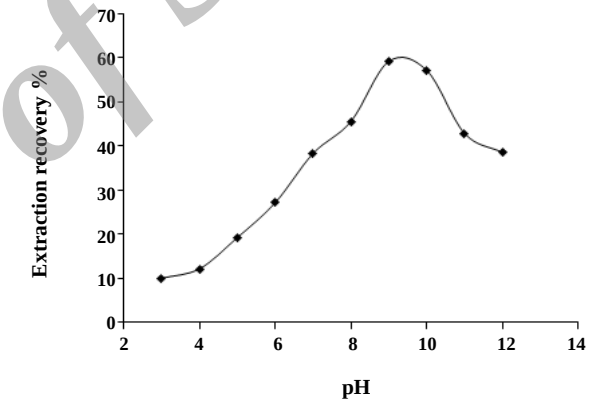

Fig. 3: Influence of sample pH on DLLME efficiency. Extraction conditions: as in Fig. 2a, except that volume of disperser solvent, aceton, is 400 µL.

To study the effect of disperser volume on the extraction recovery of crocin, all experimental conditions were fixed except the volume of methanol (0.2 to 1 mL). The results are shown in Fig. 5a. According to the obtained results, the extraction recovery increased till 0.7 mL and then decreased by increasing the volume of methanol for crocin. Therefore, a 0.7 mL volume was chosen as an optimum volume for disperser.

To examine the effect of the extraction solvent volume, solutions containing different volumes of 1-undecanol were subjected to the same DLLME-SFO procedures.

Table 2: Effect of type of the disperser solvent on DLLME-SFO extraction recovery of crocin (n=3).

//////////////////////////	Methanol	Acetone	Acetonitrile	Ethanol
1- undecanol	1.43±22.14	1.22±9.58	1.51±18.75	1.32±14.24

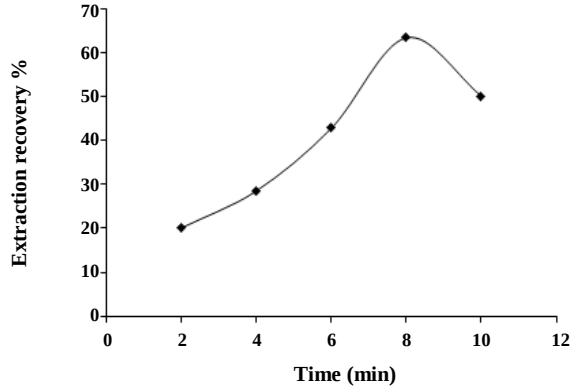

Fig. 4: Effect of centrifuge time on the extraction recovery of crocin. Extraction conditions: as in Fig. 3, except that the pH value of the aqueous solution is 9.

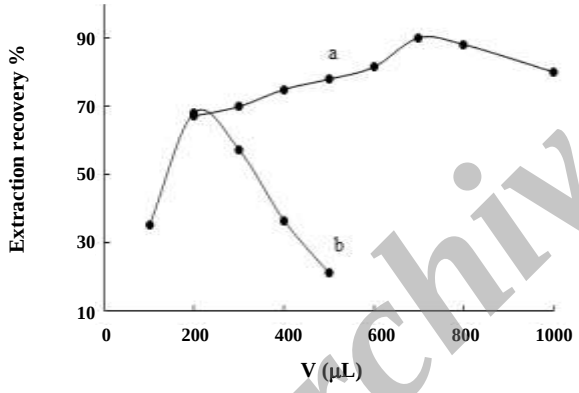

Fig. 5: (a) Effect of volume of disperser solvent, methanol on DLLME-SFO efficiency. Extraction conditions: volume of the aqueous solution, 10 mL (containing 0.1 µg/mL of crocin); and volume of 1-undecanol, 200 µL. (b) Effect of volume of extraction solvent, 1-undecanol on DLLME-SFO efficiency. Extraction conditions: as in Fig. 5a, except that volume of disperser solvent, methanol is 700 µL.

The experimental conditions were fixed and included the use of 700 µL methanol and different volume of 1-undecanol (100.0, 150.0, 200.0, 250.0, 300.0, 350.0 and 400 µL). According to the Fig.5b, by increasing volume of 1-undecanol, the extraction recovery increased to 200 µL. With the increase of extractant volume, the concentration of crocin in the sediment phase was decreased due to the dilution effect. Therefore, 200 µL was the reasonable volume for the experiment.

The effect of sample pH on the extraction of crocin was studied by varying the pH within the range of 4–12. Fig. 6 shows the influence of the sample pH on the analytical signal intensity. As it is demonstrated, the highest extraction recovery of crocin obtained at pH 9.

The addition of salt to the aqueous sample can significantly improve the extraction of several analytes in Liquid-Liquid Extraction (LLE). This is often due to the salting-out effect. In order to investigate the possibility of a salting-out effect, several experiments were performed with different NaCl concentrations (0–10% w/v). According to these results, the salt addition has no significant effect on the extraction efficiency of Cu. Therefore, all the extraction experiments were carried out without the addition of salt.

Centrifugation is a necessary step to obtain two distinguishable phases in the extraction tubes. If the centrifugation time is not enough, the organic phase cannot be completely collected on top of the vial. The effect of centrifugation time on the extraction efficiency was performed using 0.7mL of methanol and 200µL 1-undecanol added to the aqueous solution in the range of 2-10 min. The results (Fig. 7) show that the maximum extraction recovery was obtained for 6 min centrifugation time.

Quantitative analysis

To evaluate the practical applicability of the proposed DLLME and DLLME-SFO techniques, linearity, determination coefficients, limits of detection and preconcentration factors were investigated by extraction of the crocin from water samples under the optimal conditions, whose results are summarized in (Table 3).

For the purpose of quantitative analysis, calibration curves for crocin were obtained by spiking the standard directly into the distilled water and extracting under the optimal conditions by the above techniques. Linearity was observed over the range of 0.01–150 ng/mL crocin in the initial solution with the limits of detection of

Table 3: DLLME and DLLME-SFO performance and validation data.

//////////////////////////	DLLME	DLLME-SFO
Linear range (ng/mL)	0. 01-150	0. 01-150
Correlation of coefficient	0.9921	0.9917
Detection limit (ng/mL)	0. 008	0. 005
Preconcentration factor	88	95

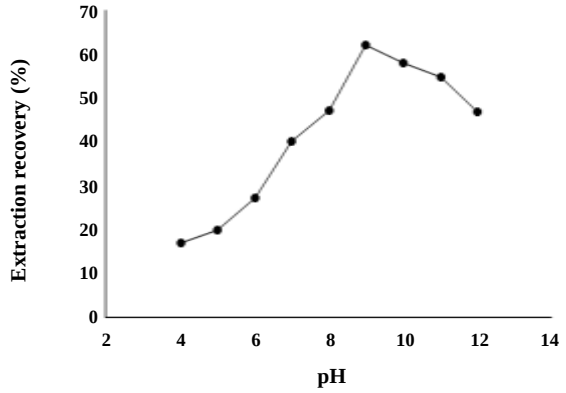

Fig. 6: Influence of sample pH on DLLME-SFO efficiency. Extraction conditions: Extraction conditions: as in Fig. 5a, except that volume of disperser solvent, methanol, is 700 µL.

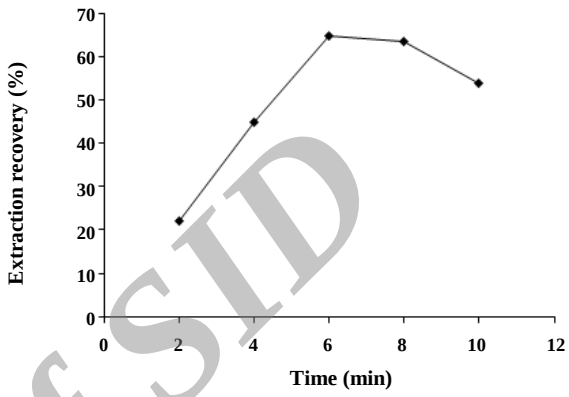

Fig. 7: Effect of centrifuge time on the extraction recovery of crocin. Extraction conditions: as in Fig. 6, except that the pH value of the aqueous solution, is 9.

0.008 and 0.005 ng/mL via DLLME and DLLME-SFO, respectively. Preconcentration factors, defined as the ratio of the slopes of the calibration curve after and before extraction, were attained 88 for DLLME and 95 for DLLME-SFO under the optimized conditions.

By comparing the data in this work with the other reported methods for crocin such as solid-phase extraction, reverse phase HPLC and indirect electrochemical analysis, it is clear that detection limit for the proposed method is better than to those obtained with other methods [22-27]. Furthermore, most of these methods are labor-intensive, time-consuming, and require a large volume of samples and organic solvents.

Analysis of real samples

To verify the applicability of the developed methods, the extraction, and determination of crocin in three different saffron samples (Ttorbatheydariyeh, Ghaen and Bakharz) and biological samples (urine and milk), were performed. All the samples were spiked with crocin standard at 50 ng/mL; subsequently, they were extracted using the DLLME and DLLME-SFO techniques and finally the extracts were analyzed by UV- Vis method.

For determination of crocin in human milk and urine, 0.5 mg of saffron was prescribed to a 28-year-old healthy nursing mother. Milk sample (3.0 mL) was collected just before and at 6 h after administration, as well as urine sample. After hydrolysis and filtration, determination of crocin was performed by standard addition method.

For each concentration level, three replicate experiments with the whole analysis process were made and experimental results are shown in Table 4. Relative recovery (RR) was calculated as follows:

$$RR(\%) = \frac{C_{spiked} - C_{unspiked}}{C_{added}} \times 100 \quad (4)$$

Where C_{spiked}, $C_{unspiked}$ and C_{added} represent the concentration of the analyte after adding a known amount of standard to the real sample, the concentration of the analyte in the real sample and the concentration of a known amount of standard that was spiked in the real sample, respectively. These results proved that the different matrices of saffron and biological samples employed in this experiment had little effects on the proposed methods.

Table 4: The application of DLLME and DLLME-SFO for determination of crocin in the saffron and biological samples (n=3).

			DLLME			DLLME-SFO	
samples	Spiked (ng/mL)	Found (ng/mL)	RSD (%)	Recovery (%)	Found (ng/mL)	RSD (%)	Recovery(%)
Saffron of Torbat-e-Heydarieh	-	250	3	-	258	2.8	-
Saffron of Torbat-e-Heydarieh	50	310	3.2	98.2%	314	3.3	99.1%
Saffron of Ghaen	-	210	2.2	-	214	2.1	-
Saffron of Ghaen	50	250	2.3	97.1%	268	2.5	98.1%
Saffron of Bakharz	-	190	2.1	-	183	2.4	-
Saffron of Bakharz	50	250	3.1	97.3%	245	3.2	97.2%
Milk	-	4.02	3.8	-	3.81	4.1	-
Milk	50	57.0	4.1	96.5%	55.2	3.9	96.8%
Urine	-	1.53	4.1	-	1.01	4.4	-
Urine	50	52.7	4.5	96.4%	51.8	4.2	96.4%

CONCLUSIONS

In this study, two liquid phase microextractions, namely, DLLME and DLLME-SFO were compared to determine the crocin in saffron and biological samples. The results confirmed that both extraction methods exhibited good linearity, precision, preconcentration factor, and the detection limit for extraction of the analytes from samples. In addition, both methods were simple, sensitive and inexpensive, and allowed sample extraction and preconcentration to be done in a single step. Moreover, DLLME-SFO was faster and the toxicity of the extraction solvent (1- undecanol) in this method is lower than that of DLLME (dichloromethane). In DLLME-SFO there is no need to use a conical bottom glass tubes in DLLME, which are easily damaged and difficulty cleaned. One of the most significant disadvantages of DLLME-SFO was the high consumption of toxic dispersive solvent (700 µL methanol) compared with DLLME (400 µL acetone) that possessed such advantages as low cost and low organic solvent consumption

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