

## Research Article

# Application of Dispersive LLM and DLLM Based on Solidification of Floating Organic Drop for the Preconcentration of Crocin in the Saffron Samples

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## Abstract

Two approaches based on dispersive liquid-liquid micro-extraction (DLLME) and dispersive liquid-liquid micro-extraction based on solidification of floating organic drop (DLLME-SFO) were compared for the extraction and pre-concentration 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 ngmL<sup>-1</sup> for DLLME and DLLME-SFO, respectively. The pre-concentration 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.

**Keywords:** Dispersive liquid-liquid micro-extraction, dispersive liquid-liquid micro-extraction based on solidification of floating organic drop, crocin, saffron, biological samples.

## Introduction

Digentiobiosyl 8, 8'-diapocarotene-8, 8'-oate; C<sub>44</sub>H<sub>64</sub>O<sub>24</sub> 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 Karree (1932) though, its presence was reported by Aschoff as long ago as the 19th century is the main pigment in the saffron (approximately, 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 scavenge free radicals, especially superoxide anions and as such may protect cells from oxidative stress.

Crocin is useful as sperm cryoconservation and in protecting hepatocytes from toxins. Because of its powerful antioxidant activity, it could be useful in the therapy of neurodegenerative disorders (Manuchair, year; Shinji et al., 2007)

UV-vis spectroscopy was widely used for 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) (Foster et al., 1993) and solid-phase extraction (SPE) (Jönsson and Mathiasson, 1999) are important techniques for extraction of analytes from liquid samples. Recently, several different types of liquid-phase microextraction (LPME) methods have been developed, including dispersive liquid-liquid microextraction (DLLME) (Rezaee et al., 2006) and dispersive liquid-liquid microextraction based on solidification of floating organic drop (DLLME-SFO) (Leong and Huang, 2008). 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) (Rezaee et al., 2006; Pereira et al., 2013; Soares et al., 2013). The main characteristics of DLLME are simplicity, low cost, suitable analyte recovery, high preconcentration factors, low solvent consumption and short time for extraction (Rezaee et al., 2010).

DLLME-SFO use solvents with the densities lower than water and lower toxicity as extractant solvent. Since, the melting point of extraction solvent is low (in the range of 10 to 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 (Lopez-Garcia and Rivas, 2010). 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 SFODME. In this method, there is no need to use conical bottom glass tubes, which are easily damaged and difficult to clean. 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 DLLMESFO for the pre-concentration 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

## Materials and Methods

**Chemicals and reagents** All reagents were of analytical reagent grade and deionized water was used throughout. Stock solution (1000 mgL<sup>-1</sup>) 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 diluted with deionized water to obtain working standard solution with a concentration of 0.1 µg/ml. Other reagents including 1-undecanol, carbon tetrachloride, dichloroethane, dichloromethane, chloroform, Orthoxylene, acetone, ethanol and acetonitril were purchased from Merck. The pH of solutions was adjusted by NaOH (0.5 mol L<sup>-1</sup>) and dropwise addition of HCl (0.5 mol L<sup>-1</sup>). Sodium chloride (Merck) was of the highest purity available.

## Extraction procedure

### **Dispersive liquid–liquid micro extraction**

A 10 ml of a standard solution or real sample and the pH was adjusted to 9 and then placed into 10 ml test tube and a mixture of 200  $\mu$ l dichloromethane as extraction solvent and 400  $\mu$ l acetone as 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 syringe and the remaining organic phase transported to a UV–vis spectrophotometer using quartz microcell to measure its absorbance at  $\lambda_{\max}$  (440 nm) for the determination of crocin.

### **Preparation of real samples**

Saffron samples obtained from different areas of Khorasan state of Iran contained 3 regions (Ttorbatheydariyeh, Ghaen and Bakharz) in the year 2015. Saffron samples were milled to make a fine powder before spectrophotometry. 50 mg of saffron was dissolved in 70 ml water slowly using magnetic shaker for 1 h and the final volume made to 100 ml. 10 ml of this sample was taken and dilution made to 100 ml. pH of the saffron sample was adjusted to pH 9 using concentrated NaOH; thereafter, 10 ml of the sample was applied to DLLME as described. Milk and urine samples were pretreated by adding 3 ml methanol and 5 ml water to a 3 ml portion of the solutions and centrifuged for 20 min. After filtration, pH of the 5 ml solutions was then adjusted to pH 9.0 using NaOH and diluted to 50 ml. Then, 10 ml of the 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 ( $n_{\text{sup}}$ ). The pre-concentration factor (PF) was defined as the ratio between the analyte concentration in the supernatant phase ( $C_{\text{sup}}$ ) and the initial concentration of analyte ( $C_0$ ) in the aqueous sample. 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 interested compounds and low solubility in water and dispersive solvent should be miscible with both water and the extraction solvent (Liang and Zhao, 2009). 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  $\mu$ l) as extractants with acetone, acetonitrile, ethanol and methanol (1 ml) as dispersive solvent were tried. Figure 1 and Table 1 indicated acetone as the disperser solvent and Dichloromethane as the extracting solvent providing maximum extraction recovery of 87.86%. Therefore, acetone/dichloromethane as a suitable set for subsequent experiment was selected.

Solvent volume is a key parameter affecting the extraction kinetics and also the pre-concentration factor. To consider the effect of disperser solvent volume on extraction recovery, different volumes of acetone were tested. Therefore, the volume of the extracting solvent (dichloromethane) was fixed at 200  $\mu$ l and

the volume of acetone was changed from 200 to 1000  $\mu\text{l}$ . The represented results in Figure 2a, shows that with increasing the volume of acetone, extraction recovery first increased till it reached a maximum point at 400  $\mu\text{l}$  and then gradually decreased by further increasing its volume. It can be attributed to the fact that, at a lower volume of acetone consumption, cloudy state was not well formed 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  $\mu\text{l}$  acetone was used as optimal disperser solvent volume.

To consider the effect of the extracting solvent volume on extraction recovery, different volumes of Dichloromethane were tested. Therefore, the volume of disperser solvent (acetone) was fixed at 400  $\mu\text{l}$  and the volume of Dichloromethane was changed from 100 to 500  $\mu\text{l}$ . According to Figure 2b, it is clear that optimum level of crocin was extracted when volume of Dichloromethane was 200  $\mu\text{l}$ . Hence, 200  $\mu\text{l}$  of Dichloromethane was selected

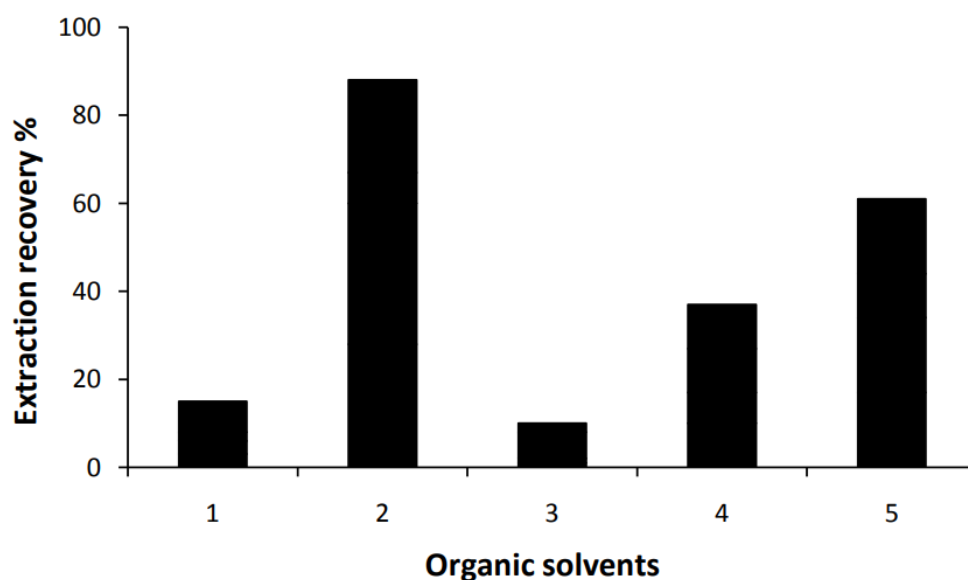


Figure 1. Effect of type of extraction solvent on DLLME extraction recovery of crocin (Acetone disperser solvent). 1. Chloroform; 2. Dichloromethane; 3. Ortho-xylene; 4. Dichloroethane; 5. Tetrachloridcarbon.

pH of the sample is an important factor during liquid– liquid extraction (LLE) process involving analytes that possess an acidic or basic moiety. 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, effect of pH of the solution on the amount of extracted crocin was investigated in the range of 3 to 12. Figure 2c shows that the best pH for extraction of crocin is 9 and that crocin is completely in its molecular form.

### 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 interested compounds and low solubility in water and it should have a melting

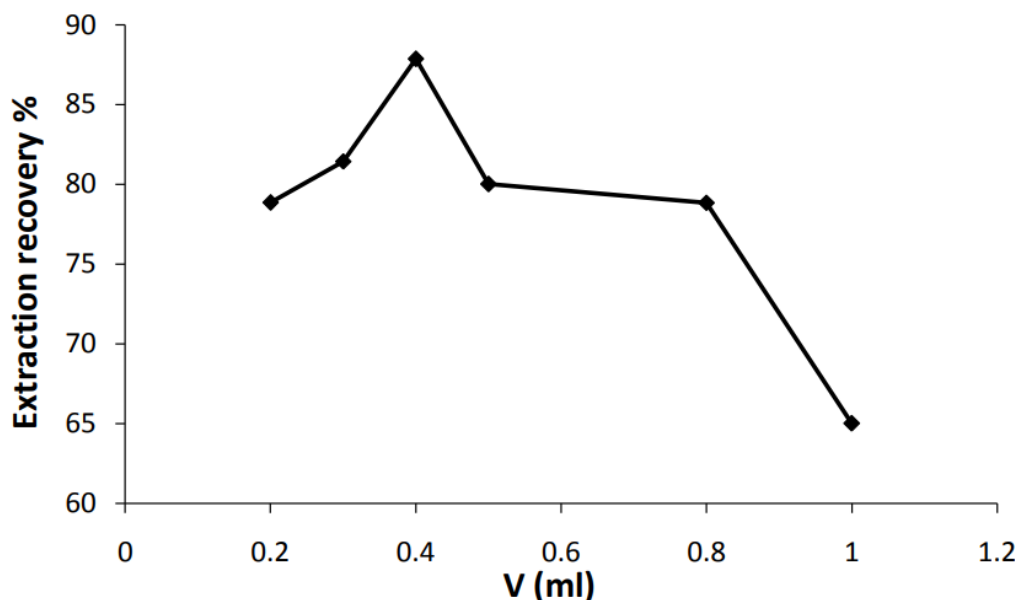


Figure 2a. Effect of volume of disperser solvent, acetone on DLLME efficiency. Extraction conditions: volume of the aqueous solution, 10 ml (containing 0.1  $\mu\text{g ml}^{-1}$  of crocin); and volume of Dichloromethane, 200  $\mu\text{l}$ .

### 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 samples. After hydrolysis and filtration, determination of crocin was performed using 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.

### 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, pre-concentration factor and detection limit for extraction of the analytes from the 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 extraction solvent (1- undecanol) in this method was lower than that of DLLME (dichloromethane).

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