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Reversed-phase Salt Assisted Liquid-liquid Extraction: A New Technique for Preconcentration and Determination of Crocin in Herbal Medicines by HPLC

Tooraj Amini and Payman Hashemi*

Department of Chemistry, Faculty of Science, Lorestan University, Khoramabad, I.R. Iran

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A reversed-phase salt assisted liquid-liquid extraction (RP-SALLE) method was developed for the preconcentration and determination of trace crocin in herbal medicines by high-performance liquid chromatography (HPLC). In developed method, the roles of the aqueous and organic phases are inverted so that the analyte is extracted from the organic into the aqueous phase after salt addition and phase separation. In this work, a mixture of methanol-acetonitrile (1:6 volume ratio) containing the extracted crocin from the powdered sample was used as the organic phase. Upon addition of sodium chloride to a mixture of methanol-acetonitrile-water and vortexing, crocin was enriched into the aqueous phase and sedimented at the bottom. A portion of the aqueous phase containing the analyte was then injected into the HPLC column for analysis. The RP-SALLE conditions including volume ratio of the solvents, pH of sample, salt concentration, time of vortex, type of salt and temperature as variable factors were carefully optimized. Under the optimized conditions, a linear calibration graph within 0.05-100 mg l^{-1} with an R^2 exciding 0.999, a detection limit of 0.02 mg l^{-1} and a relative standard deviation of 3.5% for six replicates were obtained.

Keywords: Salt assisted liquid-liquid extraction; Reversed-phase extraction; Crocin; High-performance liquid chromatography

INTRODUCTION

Crocin is a carotenoid naturally found in the flowers of crocus and gardenia. It is a diester that is formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin (Fig. 1). Its crystals (m.p.: 186 °C) have a deep red color that form an orange solution when dissolved in water. Crocin is responsible for the golden yellow-orange color of saffron. The sugar moiety of crocin has caused evident antioxidant properties for this molecule [1-4]. It has also shown antiproliferative action against cancer cells [5-7].

A variety of analytical methods have been developed for the separation and qualitative determination of crocin [8,9]. Different analytical methods such as UV-Vis spectrophotometry [10], thin layer chromatography [11], gas chromatography-mass spectrometry [12] and high performance liquid chromatography (HPLC-UV) [13] have been developed for the analysis of crocin in natural sources. However, low natural concentration of crocin in some products and their complex matrices often make necessary a separation or preconcentration step before the final analysis.

Addition of an inorganic salt into a mixture of water and an organic water miscible solvent induces a phase separation *via* the salting-out effect. Salting-out liquid-liquid extraction or salt assisted liquid-liquid extraction (SALLE) is a separation technique that was developed by Rustum in 1989 [14]. SALLE is a technique based on LLE in which an appropriate concentration of a salt is added to a mixture of an aqueous sample and a water miscible organic solvent. Accordingly, the separation of the phases is achieved and the target solutes are extracted into the separated organic phase [15]. Some of the organic solvents used in SALLE are acetonitrile [16], acetone [17], and ethyl acetate [18]. The salts commonly used are sodium chloride [16], magnesium sulfate [19] and ammonium

*Corresponding author. E-mail: payman_hashemi@yahoo.com

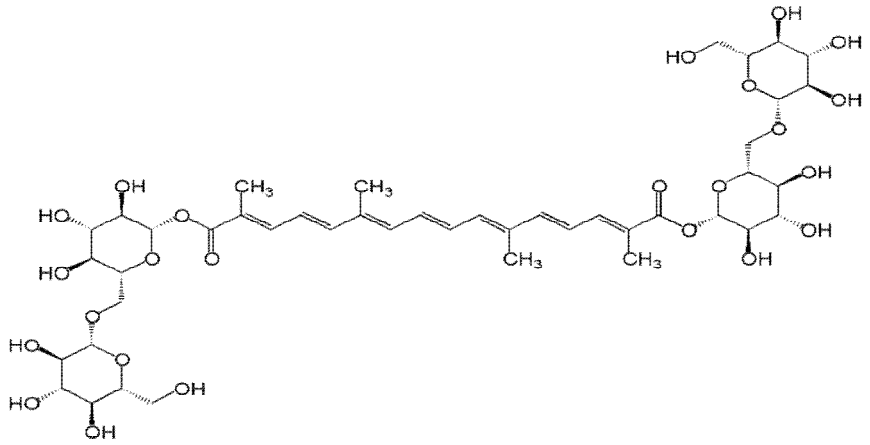

Fig. 1. Chemical structure of crocin or *trans*-crocetin di-(β-D-gentiobiosyl) ester.

sulfate [20].

The SALLE technique has been used for the extraction of a variety of compounds like drugs [21-23] and metals [24] in various sample matrices such as biological [25,26], environmental [27], food [28], plant [21] and industrial products [29].

However, this method has limitations for highly polar materials, as their extraction efficiency from aqueous phase into organic phase is low. To overcome this limitation, one possibility would be to prepare the sample in an organic phase, and then use the SALLE method to extract the polar analyte into the aqueous phase. Since in this procedure, the roles of the phases are interchanged, this method may be termed as reversed-phase SALLE or RP-SALLE.

The aim of this study is to introduce RP-SALLE as a new microextraction technique. In this method, the organic phase would be a mixture of methanol and acetonitrile as two water miscible solvents, rather than just one. Addition of methanol would increase the relative polarity of the solvent mixture providing a better condition for the initial extraction of polar analytes. The method will be used for the extraction of crocin from Aphrodite, a herbal medicine in the form of coated pills. The determination of the extracted analyte is carried out using HPLC-UV technique.

EXPERIMENTS

Reagents and Materials

Crocin was purchased from Merck (Darmstadt, Germany) and its stock solution was prepared in methanol (1000 mg l^{-1}), stored at 4 °C, and protected from light. Mixtures of diluted standard solutions were daily prepared by diluting the stock solutions. HPLC-grade methanol, acetonitrile and other chemicals were prepared from Merck (Darmstadt, Germany). Double-distilled water was used throughout. Aphrodite, a herbal medicine in the form of coated tablets, were received from Goldaru company, Isfahan, Iran.

Apparatus

The absorbance measurements were performed by Shimadzu (Japan) model UV-160 double-beam spectrophotometer. A pair of quartz 350 µl micro-cell (ES-quartz, Model Q124, Spain) was utilized for absorbance determinations of test solutions and a blank solution. Water still, Hamilton (model WSC/4D) was used for preparation of doubly distilled water. HPLC analyses were performed using a Shimadzu system consisted of two reciprocating pumps, a SPD-10A UV-Vis. detector, a high-pressure manual injection valve (with 20-µl injection loop), a DGU-14A in-line degasser and a model CT10-10AC column oven. All the injections were made by overflowing the fixed volume loop. Data acquisition and processing were performed with Lab Solution software. The separation was carried out on a 25 cm × 4.6 mm i.d. RP-18 analytical column (Shim-Pack CLCC18) packed with 5-µm particles, and equipped with a 1-cm guard column (C18-B197) packed with 10 µm particles of the same type. A 25 µl HPLC micro-syringe (F-LC, SGE, Australia) was used for the sample withdrawal and injection. In order to mix the

solvents a Vortex apparatus model Dragon Lab MX-F was used. Adjusting the pH of solutions was performed with a pH meter model Hanna (Germany) 211 equipped with a combined glass electrode.

Sample Preparation

Solid tablets of Aphrodite herbal medicine were purchased from local shops. They were finely powdered using an agate mortar. Then, 5 mg of the powder was transferred to a mixture of 2.05 ml of acetonitrile and 350 µl methanol, and the mixture was magnetically stirred for 4 h at 15 ºC in dark. After that, the extract was filtered through a 0.45 µm membrane filter. The filtrate was then transferred into a conical bottom tube containing 600 µl water (with pH adjusted on 8.5), and 0.12 g sodium chloride was added to the mixture. The solution was vortexed for 40 s. After a short while, two phases were separated. The sedimented phase at the bottom (volume, 300 µl) was the aqueous phase containing the extracted crocin, and the upper phase (volume, 2.7 ml) was the organic phase. In order to complete the phase separation, the mixture was kept under refrigeration at 11 ºC for 20 min. Finally, 25 µl of the sedimented aqueous phase was withdrawn by a micro-syringe and injected into HPLC-UV (Fig. 2).

HPLC Analysis

HPLC analysis was performed in a flow rate of 1.0 ml min^{-1} and a column temperature at 30 ºC. The mobile phase consisted of methanol-water-acetic acid (55:44.5:0.5) with a pH of 3.4, and the UV detection wavelength was adjusted on 440 nm for crocin detection [30]. The retention time of crocin was 10.8 min. Crocin concentration was calculated using an external calibration curve method.

RESULTS AND DISCUSSION

Influence of Extraction Solvent

Different solvent systems with characteristics such as high capacity to dissolve the analyte, easy phase separation by adding salt, and good chromatographic behavior were evaluated. In the conventional SALLE method, between two solvents used, water is the donor, and the water-miscible solvent is the acceptor phase that is separated from water by addition of a salt. After evaluation of various solvents such as ethanol, methanol, chloroform, ethyl acetate and acetonitrile, a mixture of methanol and acetonitrile (volume ratio: 1:6) were found to be the most appropriate solvent for the initial isolation of crocin from the powdered Aphrodite tablets. Addition of methanol increases the polarity of acetonitrile and improves the efficiency for the initial extraction of crocin and in combination with a third solvent (water) provides the possibility of phase separation and preconcentration upon addition of salt. After doing a number of tests, the optimized volume ratio of the solvents was obtained as 12% methanol, 68% acetonitrile, and 20% water.

Influence of Salt Concentration

Salt concentration is a very important factor in a salting-out extraction system. A successful phase separation and formation of a two-phase solvent system depends on the type and concentration of the salt added. Therefore, in the proposed SALLE method, the effect of salt type and concentration were carefully studied. The aim was to use the least amount of salt with an appropriate phase separation and recovery. Figure 3, shows the effect of sodium chloride concentration on the performance of the proposed method in the range of 10-80 mg ml^{-1}. The results indicated that in a salt concentration of 40 mg ml^{-1} the phase separation occurs and the highest efficiency is obtained. In a higher salt concentration, the ionic strength of the aqueous phase drastically increases that reduces the extraction efficiency into this phase due to the salting out effect. Some other salts such as sodium nitrate, potassium chloride and sodium sulfate were also tested. However, none of the salts showed a superior extraction efficiency than NaCl, and, therefore, the use of this salt continued in subsequent experiments. An explanation for the superior effect of NaCl would be the smaller size and charge of its anion and cation compared to the other used salts that reduces its salting-out effect. It should be noticed that in an RP-SALLE method, a lower salting-out effect is an advantage because the analyte is going to be extracted into the aqueous rather than the organic phase.

Effect of Sample pH

In SALLE, the sample pH is a parameter that plays a significant role as it affects the extent of the ionization as

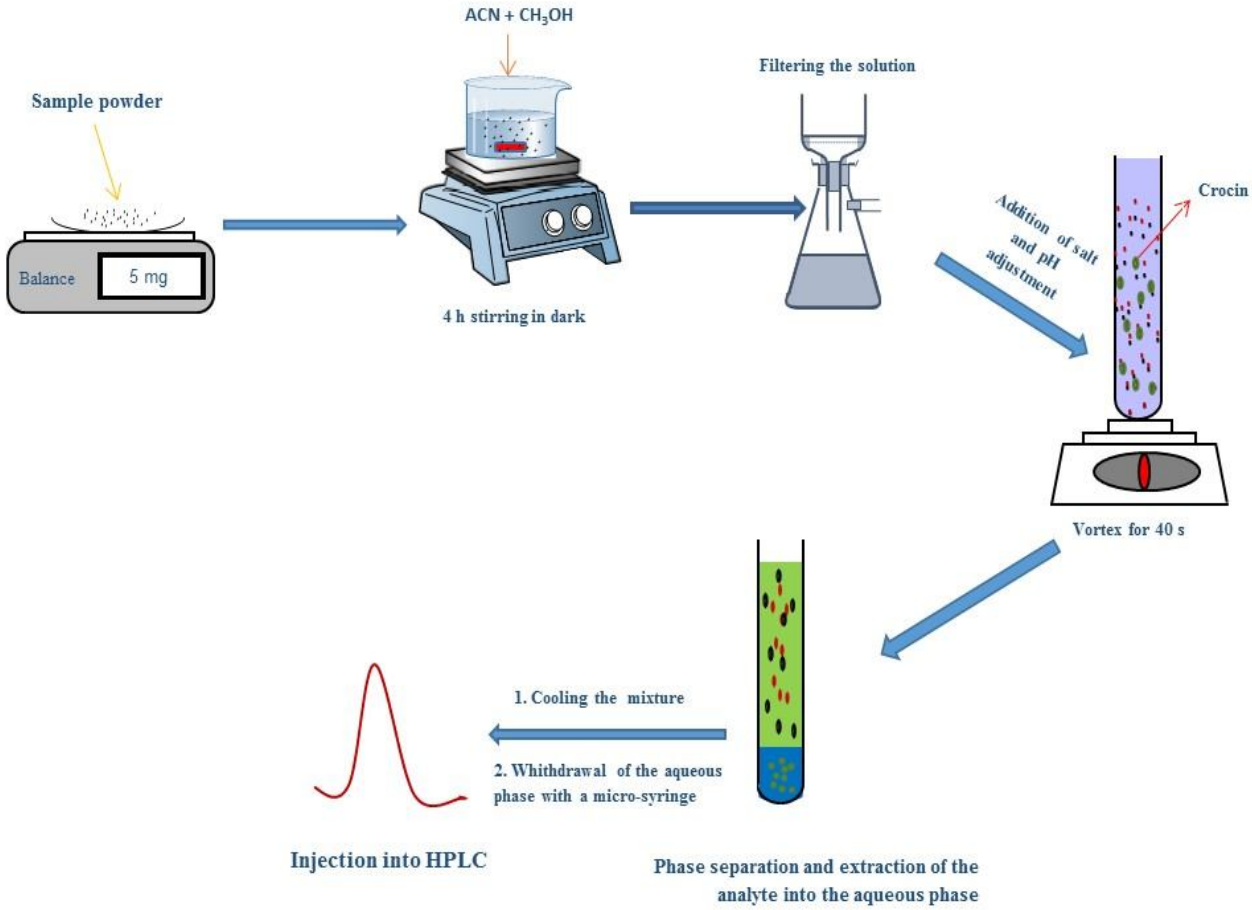

Fig. 2. Schematic illustration of the RP-SALLE procedure.

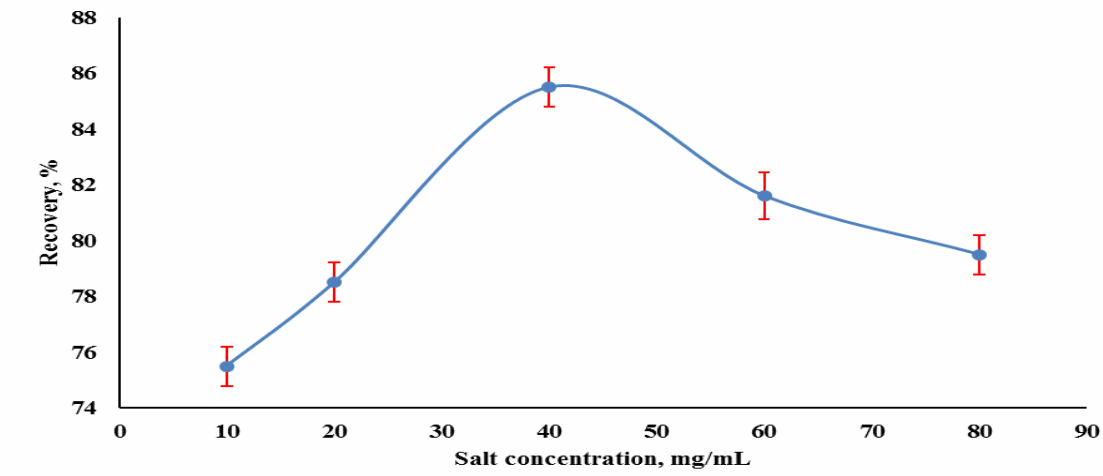

Fig. 3. Effect of salt concentration on the extraction recovery of crocin (pH = 7, Vottex time = 20 s; temperature = 25 ºC).

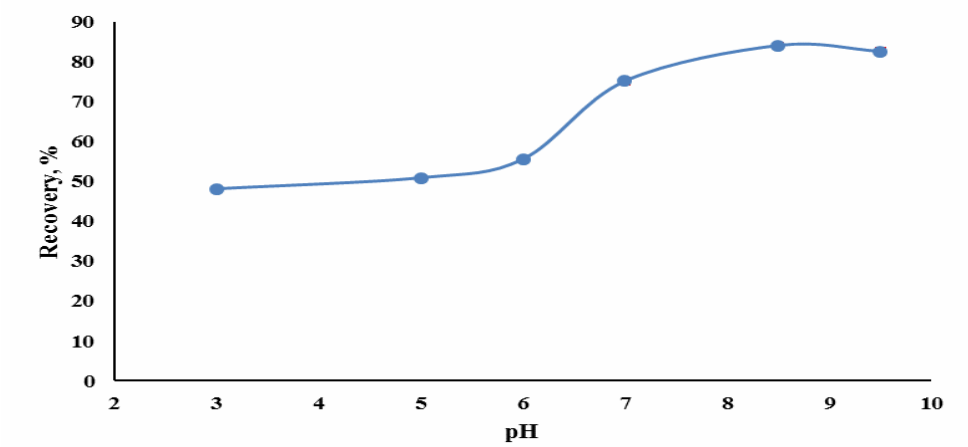

Fig. 4. Effect of sample pH on the extraction recovery of crocin (Vortex time = 20 s, Temperature = 25 ºC, Salt concentration = 40 mg ml^{-1}).

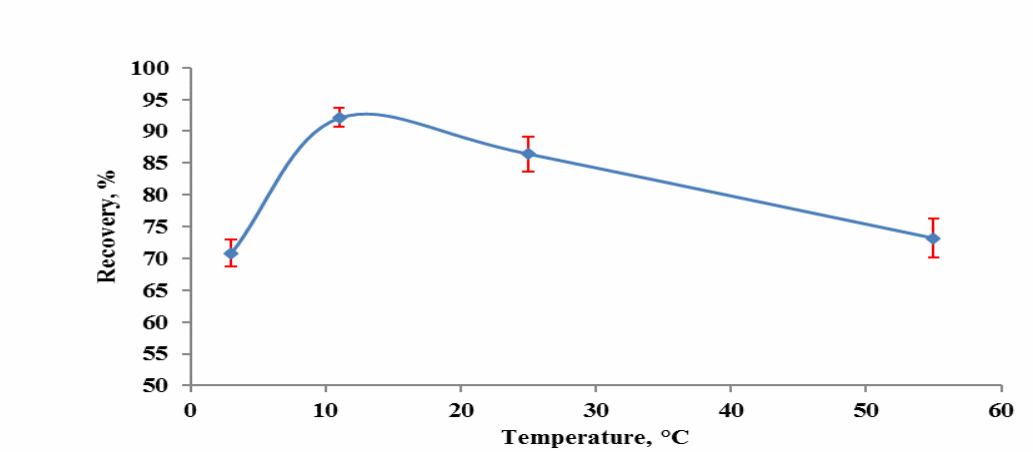

Fig. 5. Effect of temperature on the extraction recovery of crocin (pH = 8.5, salt concentration = 40 mg ml^{-1}, vortex time = 20 s).

well as the solubility of the ionisable organic compounds. It was found that the use of buffers reduces the recovery, therefore, HCl and NaOH diluted solutions were used for adjusting the pH. Effect of pH on the extraction efficiency was evaluated in the range of 3-9.5. Up to pH of 6, the change in the recovery was insignificant but after that an increase in the recovery was observed by increasing the pH up to 8.5 with its leveling off in higher pH values (Fig. 4). Crocin is a diester, and change of pH may influence the charge distribution or the extent of hydrogen bonds in its molecule and hence, influences its solubility.

Effect of Temperature

The temperature of the solution influences the amount of salt dissolved in aqueous extraction phase, and, therefore, it could be an important parameter in the RP-SALLE method. After salt addition to the mixed solvents and phase separation, the mixture was set on different temperatures of 3 to 55 ºC for 20 min. As shown in Fig. 5, at a temperature of 11 °C the highest efficiency was obtained for the extraction of crocin.

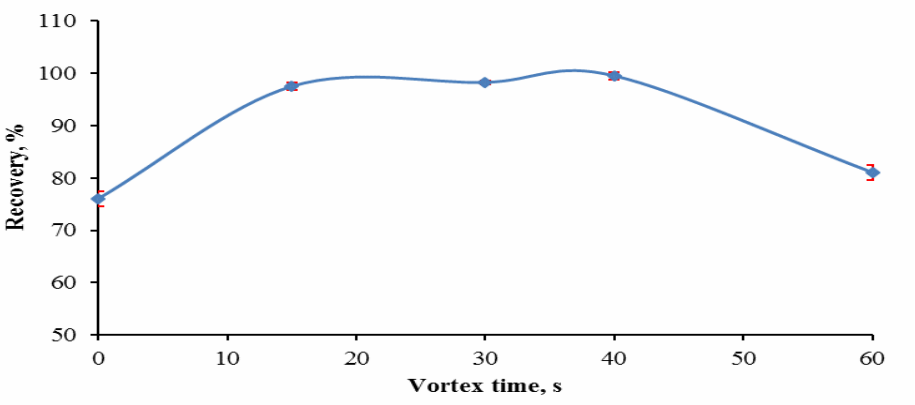

Fig. 6. Effect of vortex mixing time on the extraction recovery of crocin (pH = 8.5, salt concentration = 40 mg ml^{-1}, temperature = 11 °C).

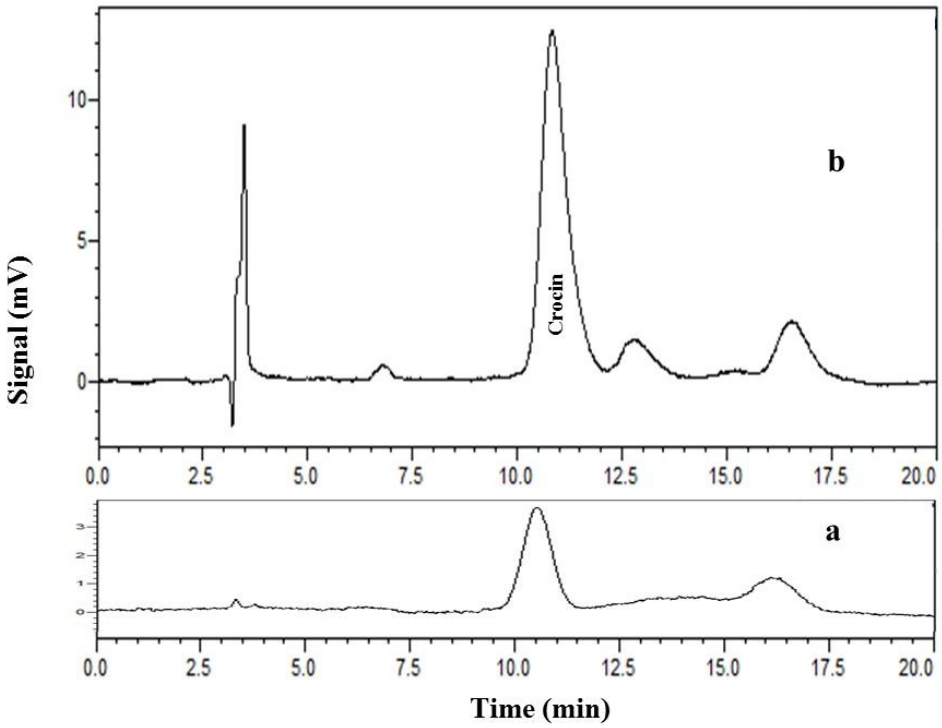

Fig. 7. HPLC chromatograms of Aphrodite sample, (a) before and (b) after the extraction and preconcentration by RP-SALLE method.

Effect of Vortex Mixing Time

Vortexing was used for dissolution of the powdered salt added to the solvent mixtures. The time of vortex mixing was found to be an important factor in the volume of phase separation and extraction efficiency. Vortex times between 0 to 60 s were tested. As shown in Fig. 6, after a minimum time of 15 s the extraction recovery remained more or less unchanged. However, 40 s vortex mixing was usually used

Table 1. The Figures of Merit and Analytical Data for the RP-SALLE Method

Analyte	LOD	LOQ	Linear range	RSD, n = 6	R^2	EF
Crocin	0.02 μg ml^{-1}	0.05 μg ml^{-1}	0.05-100 μg ml^{-1}	3.5%	0.9993	9

Table 2. Comparison of the Proposed Method with Various Extraction Techniques Used for the Extraction and Analysis of Crocin

Method	Extraction time	Organic solvent consumption	RSD	LOD	PF	Linear range	Ref.
TLC	1 h	15 ml	NR[a]	NR	-	NR	[11]
HPLC	2 h	5 ml	11%	0.2 μg ml^{-1}	-	NR	[13]
HPLC	24 h	10 ml	5%	NR	-	0.015-1 mg ml^{-1}	[31]
RP-SALLE	4 h	2.4 ml	3.5%	0.02 μg ml^{-1}	10	0.05-100 μg ml^{-1}	This work

[a]NR: Not reported.

for safety.

Method Evaluation

Under the optimaized operating conditions; *i.e*., mixed solvents of methanol (12%), acetonitrile (68%) and water (20%), NaCl concentration 400 mg l^{-1}, pH 8.5, solution temperature 11 °C, and vortex mixing time 40 s, the analytical performances of the RP-SAALE method were tested. The calibration curve was plotted in accordance with the proposed procedure using standard solutions of crocin with 9 concentration levels in the range of 0.05-100 mg l^{-1}. The calibration curve was linear within the studied range with an R^2 of 0.9993. The limit of detection (LOD) based on 3σ was equal to 0.02 mg l^{-1}. The limit of quantification (LOQ) calculated according to 10σ was 0.05 mg l^{-1}. The precision based on the relative standard deviation (RSD) of the peak area for a 1 mg l^{-1} solution of crocin was calculated to be 3.5% for six replicates. The enrichment factor (EF) defined as the ratio of the analyte concentration in the aqueous extraction phase and its initial concentration in the sample was 9. The statistical and analytical data have been summarized in Table 1.

Determination of Crocin in Aphrodite Tablets

Figures 7a and b show chromatograms corresponding to 5 mg powdered tablets of Aphrodite in 3 ml of the solvent mixture before and after the extraction and preconcentration by the RP-SALLE method, respectively. The amount of crocin in the tablets was calculated to be 6.02 (±0.2) mg g^{-1} of the sample.

In order to evaluate the accuracy of the RP-SALLE method, crocin content of Aphrodite tablets was also extracted and determined by another method reported by Caballero-Ortega *et al*. [31]. In the reported technique, a solvent extraction method with 24 h extraction time has been used following by centrifugation and HPLC determination of the analyte. Using this method, a concentration of 4.97 (±0.2) mg g^{-1} was obtained for the

Aphrodite sample. Comparison of the two methods indicated that RP-SALLE was a much faster extraction method with 9 times preconcentration of the analyte and an even higher extraction efficiency than the reported method.

CONCLUSIONS

RP-DLLME, by inverting the roles of the organic and aqueous phases, provides a wider range of applications for SALLE. This method is more appropriate for the extraction and preconcentration of analytes with high solubility in water (such as crocin). Using the minimum amounts of organic solvents would be another advantage for this innovated method. In Table 2, compares some characteristics of the proposed method with some other extraction techniques. As shown in this table, RP-DLLME has a low detection limit and reduced consumption of organic solvents. The method indicated its ability to extract and preconcentration trace amounts of crocin in herbal medicines with high efficiency and good precision.

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