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Extraction and Purification of Crocin from Saffron Stigmas Employing a Simple and Efficient Crystallization Method

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Abstract: In this study, total crocin was extracted from saffron stigmas using crystallization method. Ethanol 80% was selected as the best extraction solvent. Crystallization process was carried out in one and two steps at different temperatures. Ethanol 80% was used as crystallization medium. Crocin crystals obtained from the first crystallization had low purity and thus were subjected to the second crystallization. The higher purity crystals were yielded in the second crystallization at -5°C. The purity of crocin crystals was studied using UV-visible spectrophotometery and HPLC in comparison with Fluka product and methanolic extract of saffron stigmas. The results indicated that its purity was extremely higher, about 13 times, more than Fluka product. In spite of our expectation, the Fluka product was not a pure "-crocin sample; five other types of crocins in addition to an unknown impurity were seen in its chromatogram. The purity of crystallized total crocin in this work was more than 97%.

Key words: Crocin, saffron, extraction, crystallization, purity

INTRODUCTION

Saffron is the dried stigmas of *Crocus sativus* and the most expensive spice used in industry, with many different uses as drug, textile dye and culinary adjunct. It is mainly valued as a food additive for tasting, flavouring and colouring, as well as for its therapeutic properties. Saffron is cultivated in countries such as Iran, Spain, Italy, Switzerland and India (Sujata *et al*., 1992). It is now used worldwide in folk medicine and is reputed to be useful in treating various human disorders (Ochiai *et al*., 2007). The saffron and its colored carotenoids (crocins) have memory improving properties (Pitsikas *et al*., 2007), anticonvulsant (Hosseinzadeh and Sadeghnia, 2007), antidepressant (Akhondzadeh *et al*., 2004; Moshiri *et al*., 2006; Wang *et al*., 2009), antioxidant (Goyal *et al*., 2010; Mousavi *et al*., 2010), antitumor (Nair *et al*., 1991; Molnar *et al*., 2000; Dhar *et al*., 2009) effects.

Crocins are water soluble compounds in saffron stigmas. They belong to a family of mono- and di-glycosyl esters of polyene dicarboxylic acid named crocetin (Lozano *et al*., 1999). The "-crocin, digentiobiosyl ester of crocetin is the major fraction.

A variety of analytical methods have been developed for separation and qualitative studies of crocins (Zhang *et al*., 2004; Carmona *et al*., 2006). Different analytical methods such as, UV-Visible spectrophotometry, TLC, GC-MS and HPLC have been developed to analyze saffron. However, extraction and purification of its constituents is more important for pharmacological studies and therapeutic uses. By use of these methods crocins have been separated to several fractions (Corradi and Micheli, 1979; Sujata *et al*., 1992; Tarantilis *et al*., 1995; Lozano *et al*., 1999; Zhang *et al*., 2004). Most of these studies have been focused on analytical and determination of the structure of the crocins, but high purity crocins have not been obtained (Zhang *et al*., 2004).

Separation of crocins as an anti-cancer and antioxidant ingredient would be useful commercially and clinically. In pharmaceutical industry, crocins could be used as tablets and other dosage forms. Due to these reasons, introducing and establishing a simple and effective method for extraction of crocins from its natural sources would be necessary and useful.

In the present study, a simple and efficient method has been developed for extraction and crystallization of crocins from saffron. By the use of this process no preparative chromatography is needed. The procedure was cost effective and crocins with high yield was

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obtained. Our crystallized sample was analyzed by spectrophotometry and HPLC method in 440 nm (λ_{max} of crocin), 250 nm (λ_{max} of picrocrocin) and 308 nm (λ_{max} of safranal). Its purity has been evaluated in comparison with Fluka product and methanolic extract of saffron dried stigmas.

MATERIALS AND METHODS

This study was carried out from 2009 to 2010 at Mashhad University of Medical Sciences, Iran.

Chemicals: The "-crocin (product no. 17304) obtained from Fluka and safranal (product no. w338907) was purchased from Sigma-Aldrich (Zwijndrecht, Netherlands); saffron stigmas were purchased from Novin Saffron Co. (Mashhad, Iran). Ethanol 96% obtained from Parsian Co. (Shiraz, Iran). All the solvents used in chromatography analysis were of HPLC grade and purchased from Merck.

Extraction of crocins from saffron stigmas: Saffron stigmas powders (10 g) were suspended in 25 mL Ethanol 80% at 0°C and shaked by vortex for 2 min. After centrifugation at 4000 rpm for 10 min the supernatant was separated. Twenty five mililiter of ethanol 80% was added to sediment and extraction was repeated again. This step was repeated 6 other times. The total volume of solvent consumption for 10 g saffron stigmas in extraction process was 200 mL (8×25 mL). The resulting solution was kept in a thick walled glass container at -5°C for 24 days in darkness. The container was sealed in this period. The obtained crystals were separated from solution and washed with acetone to remove remaining water. The yielded amount of crystals was 1.7 g. In the next step, the obtained crystals were dissolved in 120 mL ethanol 80% and kept at -5°C in darkness for 20 extra days for re-crystallization. The final amount of yielded crystals was 1.02 g.

Sample preparation for analysis: The stock solutions of each sample were prepared in methanol (1 μg mL^{-1}) and were stored at 0°C in darkness. They were further diluted in water to 2 and 50 μg mL^{-1} for Spectrophotometry and HPLC analysis, respectively. For saffron stigma extract, solution was centrifuged at 13000 rpm and supernatant was kept and used as the stock.

Spectrophotometry and HPLC: The UV-Visible spectrophotometry measurements were carried out using a UV-1700 pharmaspec (Shimadzu, Japan) system in the range of 200-600 nm. Chromatographic determination of components was done on a Younglin (Soth Korea) Acme 9000 system, consisting of SP930D solvent delivery module, SDV50A solvent mixing vacuum degasser, column oven CTS30, UV730 dual wavelength UV/VIS detector and ODSA C18 (4.6×250 mm, 5 μm) column. The data analysis was done by Autochro-3000. The injection volume and flow rate were 20 μL and 0.5 mL min^{-1}, respectively. A gradient method was used for chromatographic determination of crocin (λ_{max} = 440 nm) and picrocrocin (λ_{max} = 250 nm) which the mobile phase composition was changed from 20 to 80% acetonitrile in water at 20 min. For analysis of safranal (λ_{max} = 308 nm), an isocratic method was used. The composition of mobile phase was 76% acetonitrile in water.

RESULTS AND DISCUSSION

Most earlier study on separation of crocins from saffron focused on analytical methods including TLC, HPLC and HPTLC (Tarantilis *et al*., 1995; Lozano *et al*., 1999; Zhang *et al*., 2004). Methods and high purity crocins were obtained by repeated preparative chromatography procedures. Methods reported for the later include aluminum oxide column chromatography (Escribano *et al*., 1996), TLC (Kyriakides and Skubas, 1990), gel column chromatography (Chen *et al*., 2007) and multilayer coil countercurrent chromatography, with silica gel, aluminum oxide and Sephadex LH-20 as stationary phases. These methods are tedious and have low yield. There was no previous report on application of crystallization method for separation of crocins from saffron. This method in comparison with above mentioned methods was found to be simple, efficient and obtained crocins with high purity.

Crystallization: Crystallization is the (natural or artificial) process of formation of solid crystals precipitating from a solution, melts or more rarely deposited directly from a gas. Crystallization is also a chemical solid-liquid separation technique, in which mass transfer of a solute from the liquid solution to a pure solid crystalline phase occurs. The crystallization process consists of two major events, nucleation and crystal growth. It happens in low temperature and supersaturation condition.

In earlier studies ethanol 80% have been used for extraction of crocins from saffron (Wang *et al*., 2009). Crocins are soluble carotenoid esters in this solvent at room temperature. Other compounds in saffron stigmas are insoluble or completely soluble at this condition. Therefore, ethanol 80% was applied for extraction of crocins. Insoluble compounds were separated from ethanolic extract by centrifugation. The crystallization

process was applied to separate crocins from completely soluble impurities in supernatant. The temperature was an important item for the crystallization. Different temperatures were tested. At lower temperatures than -5°C, the nucleation and crystal growth occurred faster but the purity and quality of product decreased. On the other hand, at higher temperatures crystallization process was impractically slow, with a low yield. The HPLC analysis of obtained crystals showed the higher purity of prepared crystals at -5°C. Therefore, -5°C was selected as the optimum temperature.

The amount of obtained crocin from the first crystallization step was 17% of the stigmas powder but its purity was about 85%. To increase the purity of crocins a second crystallization step was done. The results indicated that the purity of total crocin after re-crystallization was more than 97% and the amount of obtained crocins from the initial stigmas powder was 10%.

Spectrophotometry: The rank order of UV-Vis absorbance of sample solutions in spectrophotometry was as follows: Crystallized crocin > Saffron stigma extract> Fluka crocin. The data showed that, the UV absorbance of Fluka crocin (Fig. 1a) at 440 nm was 2.37 times less than saffron stigma extract sample (Fig. 1b). The absorbance of saffron stigma extract sample (Fig. 1b) was 5.6 times less than crystallized crocin (Fig. 1c) and UV absorbance of crystallized crocin (Fig. 1c) was 13.4 times more than Fluka crocin sample (Fig. 1a).

HPLC analysis: At 440 nm, six types of crocins were detected in all three chromatograms (Fig. 2a-c). In spite of our expectation, Fluka product (Fig. 2a) was not a pure "-crocin. The area under curve (AUC) of "-crocin in Fluka sample was 70% of total area of crocin types. The amount of alpha and total crocin in Fluka sample was 2.4 and 2.8 times less than saffron stigma sample (Fig. 2b). The Area under Curve (AUC) of alpha and total crocins in saffron stigma sample (Fig. 2b), were 5.77 and 4.57 times less than crystallized crocin sample (Fig. 2c). The AUC of "-crocin in crystallized sample (Fig. 2c) was 75% of total area of crocin types (Table I). The amount of "-crocin and total crocin in crystallized crocin (Fig. 2c) were 13.6 and 12.87 times more than Fluka product (Fig. 2a). This data confirms the results obtained in spectrophotometry. It meant that Fluka sample is a total crocin with a low purity in comparison with crystallized sample (Table 1).

At 250 nm (8_{max} of picrocrocin), an impurity could be seen (time = 10 min) in chromatogram of Fluka sample (Fig. 3a). At this wavelength picrocrocin peak could be detected (time = 13 min) before "-crocin in saffron extract

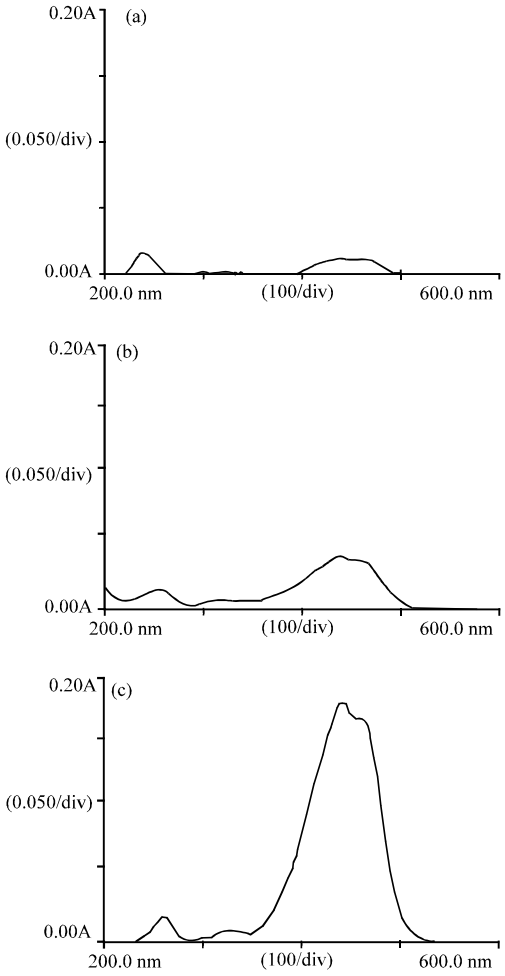

Fig. 1: Spectrophotometry of samples in water (2 µg mL^{-1}), (a) Fluka crocin, (b) saffron stigma extract sample and (c) crystallized crocin

chromatogram (Fig. 3b), but picrocrocin or any additional peak could not be detected in crystallized crocin (Fig. 3c). In Fluka sample (Fig. 3a), the AUC of the impurity, "-crocin and total crocin was 62, 4.65 and 9.36% of total AUC at this wavelength while the figures for alpha and total crocin in crystallized sample (Fig. 3c) were 75.84 and 97%. At 308 nm (8_{max} of safranal), safranal was not detected in Fluka product (Fig. 4a) while it was seen in saffron stigma extract sample (Fig. 4b). At this wavelength safranal was not detected in crystallized crocin sample (Fig. 4c).

If we assumed the Fluka product as a pure standard sample, the alpha and total crocin in saffron stigma extract would be 241 and 281% that is impossible, while if calculation was based on crystallized crocin (as a standard), the amount of alpha and total crocin would be 17.6 and 21.8% that is reasonable.

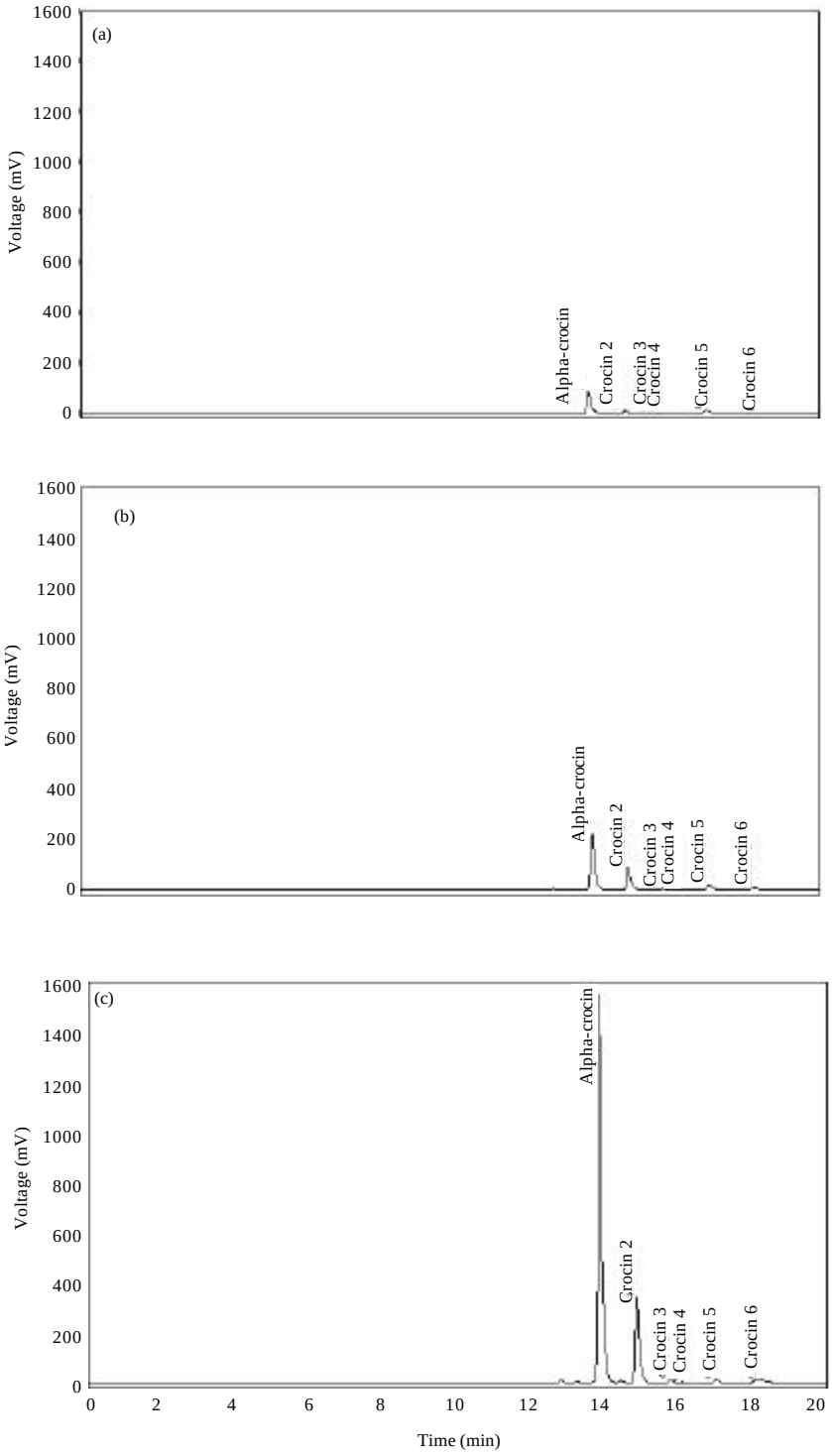

Fig. 2: Chromatograms of samples (50 µg mL^{-1}) at 440 nm, (a) Fluka crocin, (b) saffron stigma extract samples and (c) crystallized crocin. A gradient method was used which mobile phase composition was changed from 20 to 80 % MeCN in water at 20 min

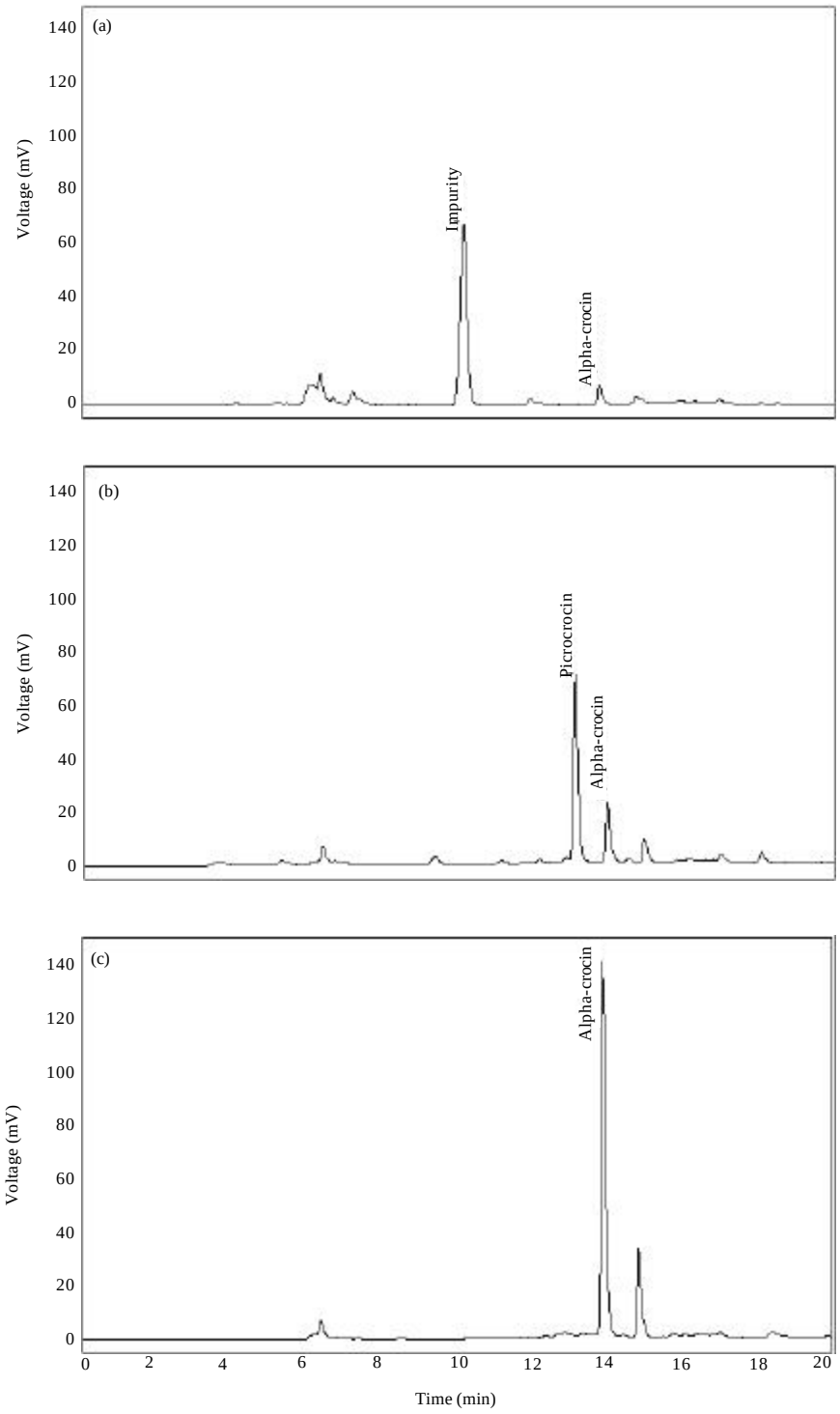

Fig. 3: Chromatograms of samples (50 µg mL^{-1}) at 250 nm, (a) Fluka crocin, (b) saffron stigma extract samples and (c) crystallized crocin. A gradient method was used which mobile phase composition was changed from 20 to 80% MeCN in water at 20 min

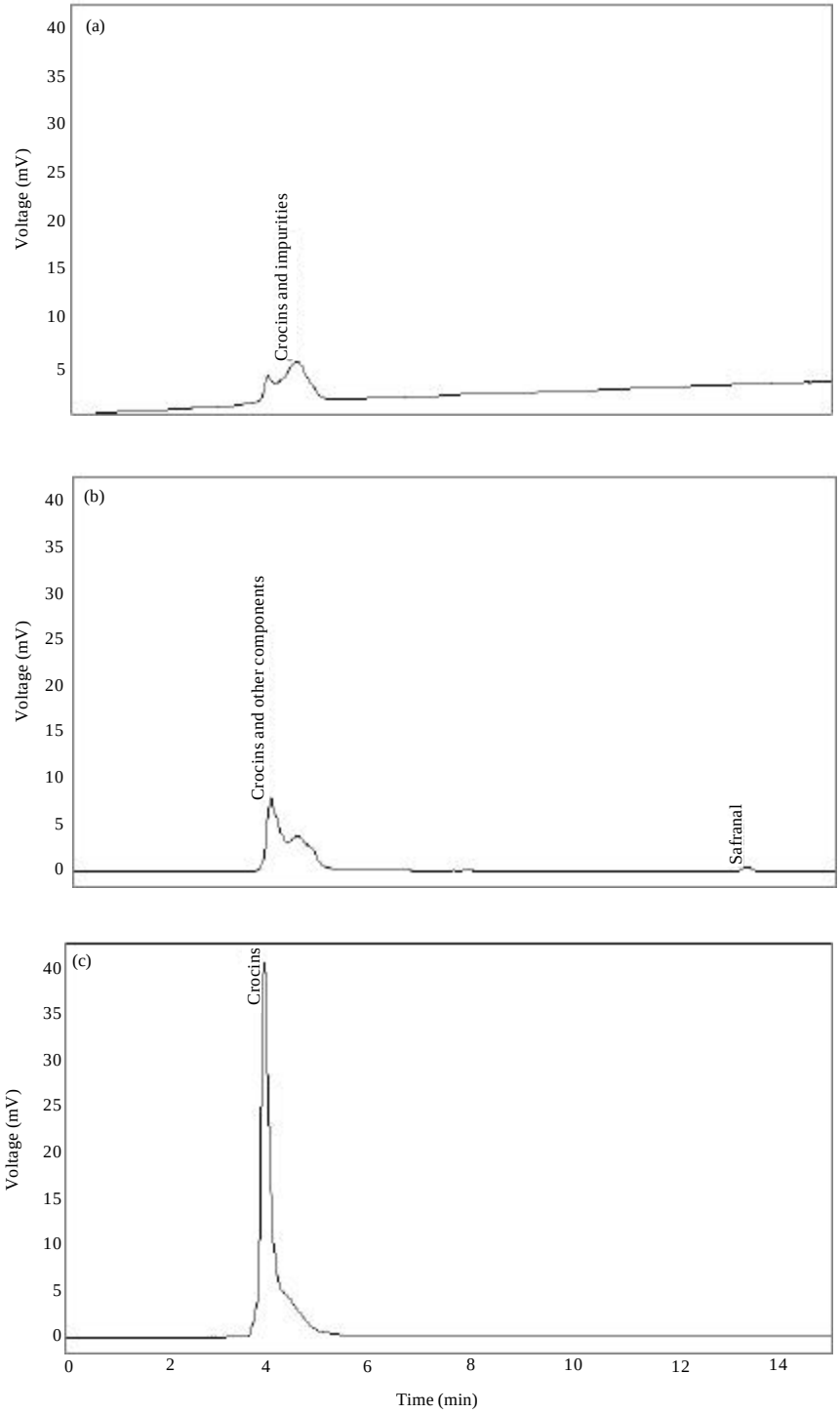

Fig. 4: Chromatograms of samples (50 µg mL^{-1}) at 308 nm, (a) Fluka crocin, (b) saffron stigma extract samples and (c) crystallized crocin. An isocratic method was used which mobile phase composition was 76% MeCN in water

Table 1: AUCs of different crocin types and percent of each peak in chromatograms of samples obtained by HPLC at 440 nm in a gradient method

		Fluka "-crocin		Stigma extract		Crystallized crocin	
Compound	RT (min)	Area (mv×sec)	Area (%)	Area (mv×sec)	Area (%)	Area (mv×sec)	Area (%)
" – crocin	13.83	715.60	70.88	1728.53	60.77	9765.10	75.15
Crocin 2	14.85	79.84	7.91	723.19	25.43	2640.48	20.32
Crocin 3	15.73	18.13	1.80	45.24	1.59	102.81	0.79
Crocin 4	16.06	15.91	1.58	32.18	1.13	22.38	0.17
Crocin 5	16.96	125.32	12.41	187.43	6.59	164.50	1.27
Crocin 6	18.13	54.84	5.43	127.71	4.49	299.45	2.30

Due to the same measurement method and fixed device sensitivity, for better comparison between Fluka product and crystallized crocin, Limit of Detection (LOD) and Limit of Quantification (LOQ) could be calculated for each sample.

At a signal/noise ratio of 3, the assay had an LOD of 53.1 ng mL^{-1} for "-crocin in Fluka product and 2.9 ng mL^{-1} for "-crocin in crystallized sample. At a signal/noise ratio of 20 - which represents 5% error in the signal-the assay had an LOQ of 345 ng mL^{-1} for "-crocin in Fluka product and 19.3 ng mL^{-1} for "-crocin in crystallized sample. As it is clear, the values of LOD and LOQ of crystallized crocin is less than Fuka product and demonstrated higher purity of our product in comparison with Fluka crocin.

These data demonstrated that the purity of crocin crystals were significantly (13 times) higher than Fluka "-crocin and can be used as a reliable standard. This study introduced a simple method for preparing quantities of pure crocin for pharmaceutical/biomedical applications and evaluation.

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