

# Simultaneous quantification of crocetin esters and picrocrocin changes in Chinese saffron by high-performance liquid chromatography-diode array detector during 15 years of storage

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## ABSTRACT

**Background:** Saffron, which is made up of the dried stigmas of *Crocus sativus* L., has been successfully cultivated in China since 1970s and Zhejiang province is now the largest producing area in China, but the contents of crocetin esters and picrocrocin in saffron from Zhejiang province has not been determined simultaneously by high-performance liquid chromatography (HPLC) and changes of these constituents in Chinese saffron during storage for years has not been studied. **Object:** To establish a simple method quantification of the five main compounds including picrocrocin and four crocetin esters in saffron from main producing areas of China and study the influence of storage time on the changes of saffron constituents. **Materials and Methods:** A simple, sensitive, and accurate HPLC method was developed for simultaneous determination of five major active components in saffron and eight samples which collected from the same farm of Zhejiang province in different years were analyzed. **Results:** The correlation coefficient values ( $R^2 > 0.9997$ ) indicated good correlations between the investigated compounds' concentrations and their peak areas within the test ranges. The limits of quantification and detection of the five compounds were 0.53–2.76  $\mu\text{g/mL}$  and 0.11–0.77  $\mu\text{g/mL}$ , respectively. The recoveries ranged from 94.67% to 101.31%, and the overall relative standard deviations for intra-day and inter-day were lower than 3.49%. The method was applied to study the changes of crocetin esters and picrocrocin contents in saffron samples during 15 years of storage. The losses of crocetin esters and picrocrocin in saffron with 1-year storage were 52.2% and 54.3%, respectively. The trend then declined during subsequent storage. **Conclusion:** The developed method can be applied to the intrinsic quality control of saffron.

**Key words:** Crocetin esters, high-performance liquid chromatography-diode array detector, picrocrocin, saffron, storage

## INTRODUCTION

Saffron, which is made up of the dried stigmas of *Crocus sativus* L., is a very expensive spice in the world. The annual production of saffron is estimated to be around 300 time/year, and Iran is the largest saffron producer in the world, accounting for more than 90% of the world's

production.<sup>[1]</sup> In China, saffron is a well-known traditional Chinese medicine and is used to stimulate blood flow and relieve pain by removing stagnated blood.<sup>[2]</sup> Saffron was imported to China from abroad, bypassing Tibet, in ancient times and has been successfully cultivated in more than 20 provinces in China since the 1970s. Zhejiang province ranks first in the Chinese production of saffron with more than 90% of the Chinese yield.

The main active compounds of saffron are crocetin esters, picrocrocin and safranal, responsible for saffron's coloring power, bitter taste and aroma,<sup>[3]</sup> respectively. The structures of the main components from saffron

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are shown in Figure 1. The levels of these active compounds are important references in quality evaluation for commercial purposes. Several methodologies have been established for the determination of the quantities of bioactive constituents of saffron, such as thin-layer chromatography,<sup>[4]</sup> gas chromatography (GC),<sup>[5]</sup> and high-performance liquid chromatography (HPLC),<sup>[6]</sup> Raman spectroscopy and chemometrics,<sup>[7]</sup> and micellar electrokinetic chromatographic.<sup>[8]</sup> Among the analytical techniques used, HPLC-diode array detector (HPLC-DAD) has been shown to be the most efficient technique for the analysis of compounds in natural products.

To our knowledge, only the trans-crocetin esters<sup>[9,10]</sup> as well as picrocrocin<sup>[10]</sup> and safranal<sup>[10]</sup> have been isolated to establish calibration curves for these quantitative determinations, and only the samples from Azerbaijan, China, France, Greece, India, Iran, Italy, New Zealand, Spain, and Turkey have been studied.<sup>[9-12]</sup> The Chinese saffron used in these studies was collected in Tibet<sup>[9,12]</sup> or Sichuan province<sup>[11]</sup> which are not the main producing areas of China.

In addition, it is also very important to study the influence of storage time on the content changes of the main compounds in saffron because of the instability of these compounds. Only the ultraviolet (UV)<sup>[13]</sup> and GC<sup>[14]</sup> methods were applied to characterize the saffron coming from different storage times. Moreover, the results determined by UV do not give an accurate measurement of picrocrocin and safranal because of the interference of cis-isomers in the crocetin esters which absorb at both 250 and 330 nm.<sup>[15]</sup> Further, there are no papers studying

content changes in saffron by HPLC after storage for longer than 3 years.

The purpose of this paper was to study the quality of saffron from the main producing areas of China and the influence of storage on the main crocetin esters and picrocrocin contents. Seven samples from main producing areas in China stored from 1998 to 2013 were collected. Then, a simple HPLC-DAD method for simultaneous determination of levels of picrocrocin and four crocetin esters, including cis-4-GG, determined by an external standard method for the first time, was established. The effect of storage on crocetin esters and picrocrocin changes in saffron could then be discussed.

## MATERIALS AND METHODS

### Plant materials

All saffron samples with different years of storage were collected from the same farm in Zhejiang province of China with the exception of the sample used in the optimization of the extraction method which was purchased in the market. In order to avoid the influence of the drying process, the dehydration of all samples was kept the same: The fresh stigma was dried by hot air at 110°C for 6 min at 6 m/s and cooled to room temperature, and the dried saffron was put into zip lock bag and kept in a cool and dry place at room temperature until chemical analysis. Detailed information on the saffron samples is shown in Table 1.

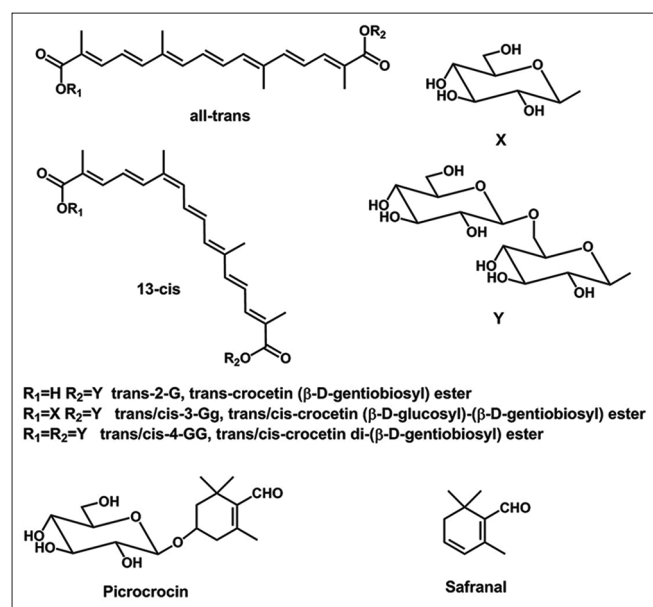
### Chemicals and reagents

Methanol and Acetonitrile (TEDIA company, USA) were HPLC-grade. The deionized water was prepared from a Millipore water purification system (Milford, MA, USA) and filtered with a 0.25 µm membrane. Other reagents were all of analytical grade. The five standard compounds, picrocrocin, trans-4-GG, trans-3-Gg, trans-2-G, and cis-4-GG [Figure 1] were purified from saffron in our laboratory. Their chemical structures were elucidated by comparing their UV and <sup>1</sup>H nuclear magnetic resonance or mass spectrum data to the reported data. The purities of all the reference compounds were more than 95%.

### Apparatus and chromatographic conditions

Sample preparation was performed using ultrasound-assisted extraction (UAE) in a KQ-500 B ultrasonic device (Kunshan Ultrasound Instrument Company, China) with a frequency of 40 kHz and an ultrasound input power of 500 W and equipped with a digital timer and temperature controller.

All chromatographic measurements were carried out on the Agilent 1260 HPLC consisting four pumps, an autosampler, and a DAD. Separation was carried out on an Agilent C<sub>18</sub> (250 mm × 4.6 mm i.d., 4 µm) column. 20 µL of the



**Figure 1:** Chemical structures of main crocetin esters, picrocrocin, and safranal from saffron

**Table 1: Quantities of the main compounds in saffron (mean (%)  $\pm$ SD, n=3)**

Number	Years of storage	Picrocrocin	Trans-4-GG	Trans-3-Gg	Trans-2-G	Cis-4-GG	Crocetin esters
S1	15	0.0314 $\pm$ 0.0004 <sup>a</sup>	ND <sup>a</sup>	0.0036 $\pm$ 0.0001 <sup>a</sup>	0.0043 $\pm$ 0.0001 <sup>a</sup>	0.0023 $\pm$ 0.000 <sup>a</sup>	0.0096 $\pm$ 0.0009 <sup>a</sup>
S2	13	0.2100 $\pm$ 0.0030 <sup>b</sup>	0.0053 $\pm$ 0.0001 <sup>a</sup>	0.0238 $\pm$ 0.0002 <sup>a</sup>	0.0104 $\pm$ 0.0002 <sup>a</sup>	0.0021 $\pm$ 0.000 <sup>a</sup>	0.0416 $\pm$ 0.0004 <sup>a</sup>
S3	10	2.1284 $\pm$ 0.0297 <sup>c</sup>	1.7170 $\pm$ 0.0397 <sup>b</sup>	0.6355 $\pm$ 0.0107 <sup>b</sup>	0.2234 $\pm$ 0.0040 <sup>b</sup>	0.2077 $\pm$ 0.0035 <sup>b</sup>	2.7836 $\pm$ 0.0309 <sup>b</sup>
S4	8	5.1443 $\pm$ 0.0975 <sup>d</sup>	4.9716 $\pm$ 0.0930 <sup>c</sup>	2.0068 $\pm$ 0.0309 <sup>c</sup>	0.5137 $\pm$ 0.0063 <sup>c</sup>	0.2264 $\pm$ 0.0030 <sup>c</sup>	7.7186 $\pm$ 0.1310 <sup>c</sup>
S5	2	6.2842 $\pm$ 0.0926 <sup>e</sup>	6.7539 $\pm$ 0.1027 <sup>d</sup>	2.4788 $\pm$ 0.0387 <sup>d</sup>	0.5794 $\pm$ 0.0008 <sup>d</sup>	0.0691 $\pm$ 0.0009 <sup>d</sup>	9.8812 $\pm$ 0.0922 <sup>d</sup>
S6	1	7.2281 $\pm$ 0.1195 <sup>f</sup>	8.2641 $\pm$ 0.0610 <sup>e</sup>	3.1566 $\pm$ 0.0515 <sup>e</sup>	0.3761 $\pm$ 0.0075 <sup>e</sup>	0.1027 $\pm$ 0.0017 <sup>e</sup>	11.8995 $\pm$ 0.0992 <sup>e</sup>
S7	0	15.8075 $\pm$ 0.1217 <sup>g</sup>	17.6646 $\pm$ 0.1019 <sup>f</sup>	5.6624 $\pm$ 0.0322 <sup>f</sup>	1.3287 $\pm$ 0.0254 <sup>f</sup>	0.2148 $\pm$ 0.0025 <sup>f</sup>	24.8704 $\pm$ 0.1007 <sup>f</sup>

Different lower letters in the columns indicate a significant difference ( $P < 0.05$ ) between samples. ND: No detected; SD: Standard deviation

**Table 2: Solvent gradient conditions for HPLC-DAD**

Time (min)	Methanol (%)	Acetonitrile (%)	Water (acidified with formic acid, 0.2%) (%)
0	10	13.5	76.5
60	100	0	0

HPLC-DAD: High-performance liquid chromatography-diode array detector

extract were injected into the chromatograph at 30°C. The solvent gradient conditions are shown in Table 2, and the flow rate was 1.0 mL/min. The DAD detector was set at a full spectrum (200–600 nm) and 440 and 254 nm for crocetin esters and picrocrocin, respectively. The five marker constituents were sufficiently resolved and successfully separated. Representative chromatograms of the saffron are shown in Figure 2.

### Preparation of standard solutions

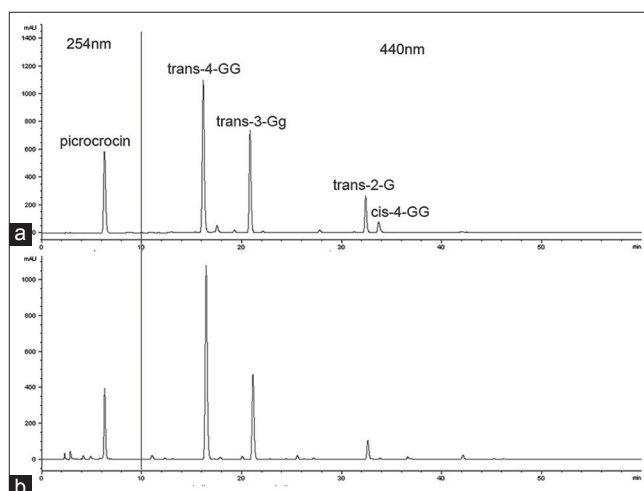
The standard stock solutions of the five compounds were prepared in methanol-water (1:1) with a concentration of 770  $\mu$ g/mL for picrocrocin, 216  $\mu$ g/mL for trans-4-GG, 109  $\mu$ g/mL for trans-3-Gg, 32.2  $\mu$ g/mL for trans-2-G and 18.2  $\mu$ g/mL for cis-4-GG. The stock solutions were serially diluted and used for preparation of working standard solutions, which were filtered through a polyvinylidene difluoride (PVDF) filter of 0.45  $\mu$ m and stored at 4°C.

### Sample preparation

Saffron was ground with an agate pestle and mortar and passed through a 0.4 mm sieve. Then 10 mg of saffron was extracted by ultrasonic-assist for 90 min in 10 mL of methanol-water (1:1) and filtered through a PVDF filter of 0.45  $\mu$ m. The sample solution was then kept at 4°C until taken out for analysis. The whole process was carried out in the darkness.

### Statistical analysis

The values obtained from analyses of the five compounds in samples were performed in triplicate and the data were expressed as mean  $\pm$  standard deviations which were calculated using Microsoft Excel Software 2007. Experimental data were also subjected to analysis of



**Figure 2:** (a) The high-performance liquid chromatography chromatogram of a standard mixture and (b) the extract of saffron

variance and significant ( $P < 0.05$ ) means were determined using Duncan's multiple-range test to distinguish differences between content means at 5% level using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSION

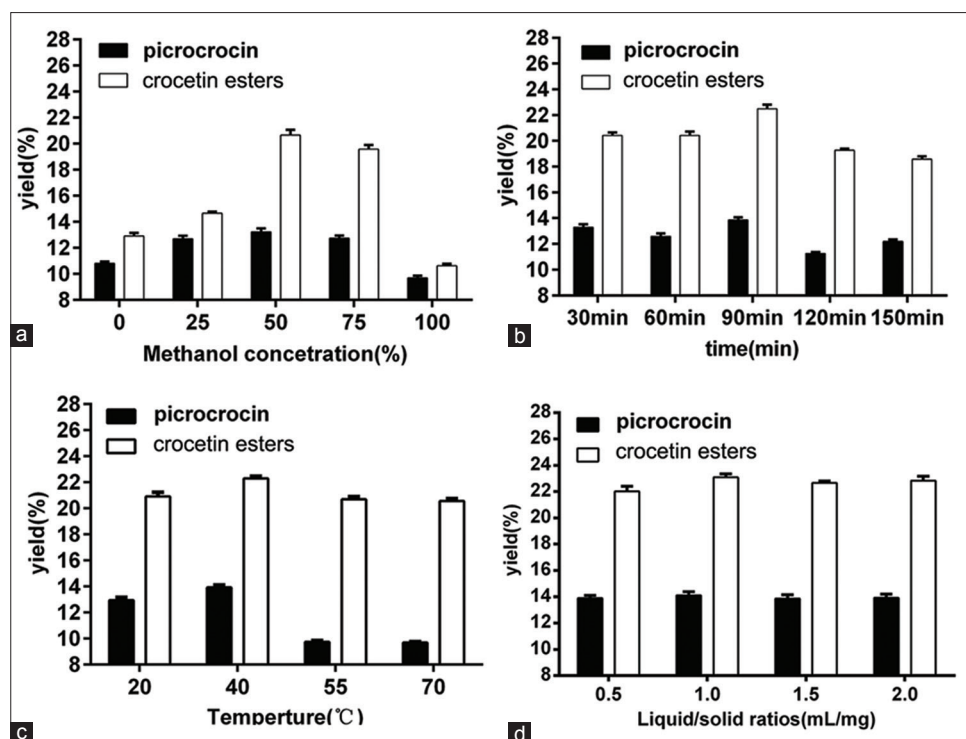
### Optimization of extraction method

To achieve the best UAE conditions, various concentrations of extraction solvents (0%, 25%, 50%, 75%, and 100% methanol), five different ultrasonic times (30, 60, 90, 120, and 150 min), increasing temperatures (20, 40, 55, and 70°C), and six liquid/solid ratios (0.5, 1, 1.5 and 2 mL/mg) were investigated for the higher yields of picrocrocin and crocetin esters (sum of trans-2-G, trans-3-Gg, trans-4-GG, cis-4-GG). The satisfactory conditions are shown in the section for sample preparation based on Figure 3.

### Validation of the developed method

#### Calibration curves, limits of detection and limits of quantification

Calibration curves of the five compounds were calculated based on the peak areas (y) of the concentrations of



**Figure 3:** The effects of the extraction parameters on yields of picrocrocin and crocetin esters: (a) Effect of methanol concentration on yields of picrocrocin and crocetin esters. (b) Effect of extraction time on yields of picrocrocin and crocetin esters. (c) Effect of extraction temperature on yields of picrocrocin and crocetin esters. (d) Effect of liquid/solid ratio on yields of picrocrocin and crocetin esters

**Table 3: Linear regression data, LOD and LOQ of the investigated compounds**

Analyte	Regression equation	$R^2$	Linear range ( $\mu\text{g/mL}$ )	LOD ( $\mu\text{g/mL}$ )	LOQ ( $\mu\text{g/mL}$ )
Picrocrocin	$Y=21.098X-26.335$	0.9997	7.71-385.00	0.77	2.76
Trans-4-GG	$Y=70.973X+5.0223$	1.0000	1.49-216.00	0.18	0.62
Trans-3-Gg	$Y=86.799X+6.5501$	1.0000	0.76-109.00	0.12	0.66
Trans-2-G	$Y=105.44X-18.3$	0.9999	0.80-32.20	0.13	0.53
Cis-4-GG	$Y=71.938X-6.6626$	0.9998	0.91-18.20	0.11	0.59

LOD: Limits of detection; LOQ: Limits of quantification

**Table 4: The intra- and inter-day precision**

Analyte	Intra-day precision		Inter-day precision	
	Mean $\pm$ SD ( $\mu\text{g/mL}$ )	RSD (%)	Mean $\pm$ SD ( $\mu\text{g/mL}$ )	RSD (%)
Picrocrocin	230.4 $\pm$ 3.4	1.48	229.8 $\pm$ 4.8	2.09
Trans-4-GG	107.1 $\pm$ 1.8	1.68	105.7 $\pm$ 2.3	2.18
Trans-3-Gg	54.04 $\pm$ 0.9	1.67	53.1 $\pm$ 1.3	2.45
Trans-2-G	16.06 $\pm$ 0.3	1.88	15.6 $\pm$ 0.5	3.21
Cis-4-GG	8.9 $\pm$ 0.2	2.25	8.6 $\pm$ 0.3	3.49

SD: Standard deviation; RSD: Relative standard deviation

the working standard solutions (x), created by diluting stock solutions in series. The regression equation and correlation coefficients ( $R^2$ ) are listed in Table 2, and the high correlation coefficient values ( $R^2 > 0.9997$ ) show good linearity at a relatively wide range of concentration. The limits of detection and quantification for each analyte under

present chromatographic conditions were determined at the signal-to-noise ratio (S/N) for each compound to be about 3 and 10, respectively. The results are shown in Table 3.

#### Precision, repeatability, and stability

The intra-day and inter-day precisions were investigated by determining levels of each reference compound at one concentration level in six replicates during a single day and by replicating the experiments on three consecutive days, with variations expressed by relative standard deviations (RSD).

To further evaluate the repeatability of the developed assay, six sample solutions were prepared and analyzed as described above. The contents of the five compounds in saffron were calculated from the corresponding



**Table 5: Analytical results of repeatability, stability, and recovery test**

Analyte	Repeatability (n=6)		Stability RSD (%)	Recovery (n=6)	
	Mean±SD (µg/mL)	RSD (%)		Mean±SD (%)	RSD (%)
Picrocrocin	12.9227±0.2407	1.8628	0.1024	98.19±2.22	2.26
Trans-4-GG	14.8417±0.2718	1.8312	0.3562	94.67±1.13	1.20
Trans-3-Gg	5.2974±0.0971	1.8337	0.3217	96.41±1.96	2.04
Trans-2-G	1.4549±0.0317	2.1808	1.1103	100.08±2.09	2.09
Cis-4-GG	0.2902±0.0055	1.8988	0.4201	101.31±2.25	2.22

SD: Standard deviation; RSD: Relative standard deviation

calibration curves. The RSD were taken as measurements of repeatability.

The stability was tested with one of the samples used in the repeatability evaluation. The solution was kept at 4°C, and 1 mL was taken out to analyze at 0, 2, 4, 8, 12, and 24 h. The results are shown in Table 4.

### Accuracy

Recovery tests were used to evaluate the accuracy of the method. The recoveries of analytes varied from 94.67% to 101.31%, and RSD values were in the range of 1.20–2.26%, demonstrating the reliability and accuracy of the measurement of these constituents. The results are shown in Table 5.

### Analysis of saffron

The HPLC-DAD method established was applied in the analysis of the five compounds in the saffron samples. The peaks of each compound in saffron were identified by comparing the retention time and UV spectra with those of the standards. The levels of the five compounds in saffron were calculated from the calibration curves of each standard. The main crocetin esters and picrocrocin quantities in the samples included in this study are shown in Table 1. The losses of crocetin esters and picrocrocin after the 1<sup>st</sup> year of storage were 52.2% and 54.3%, respectively. The trend then declined during subsequent storage. This result is similar to the work of Raina *et al.*<sup>[13]</sup> However, the changing trends of single crocetin esters were different; the levels of trans-4-GG and trans-3-Gg decreased every year whereas the levels of trans-2-G and cis-4-GG first decreased and then increased, and then both decreased.

## CONCLUSIONS

The developed method for the determination of picrocrocin, trans-4-GG, trans-3-Gg, trans-2-G, and cis-4-GG quantities was sensitive and reliable. It could be used to monitor the quality of saffron and other related

pharmaceutical preparations. Based on the data obtained, the maximum storage time of saffron from the main producing areas of China is not more than 2 years.

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