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Simultaneous quantification of five major biologically active ingredients of saffron by high-performance liquid chromatography

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Abstract

A simple, sensitive and specific high-performance liquid chromatography–UV (HPLC–UV) method has been developed for the first time to simultaneously quantify the five major biologically active ingredients of saffron, namely crocin 1, crocin 2, crocin 3, crocin 4 and crocetin. Calibration curves were derived by spiking authentic compounds and internal standard, 13-*cis*-retinoic acid, into herbal samples prior to extraction. Extraction was conducted simply by stirring dried herb (20 mg) with 80% aqueous methanol (5 ml) at ambient temperature in the dark for 2 h. The HPLC assay was performed on a reversed-phase C_{18} column with linear gradient elution using methanol and 1% aqueous acetic acid. Calibrations were linear ($r^2=0.999$) for all five analytes, with overall intra- and inter-day RSDs of less than 11%. The assay was successfully applied to the determination of four crocins and crocetin in three saffron samples and two Zhizi, another crocin-containing herb. Results indicate that the developed HPLC assay can be readily utilized as a quality control method for crocin-containing medicinal herbs.

Keywords: Saffron; *Crocus sativus*; *Gardenia jasminoides*; Crocins; Crocetin

1. Introduction

Saffron, the dried stigmas of *Crocus sativus* L., is a very expensive spice that is used mainly as a herbal medicine or food coloring and flavoring agent in different parts of the world [1–6]. Saffron originally grew in India, Iran, Spain, Greece and other countries, and has been successfully cultivated in various places in China, especially Tibet [5,6]. It has been used as an anti-anginal traditional Chinese medicine (TCM) for a long time [6]. The major biologically active ingredients of saffron are crocin analogues, including crocins 1–4, which are all glycosides of *trans*-crocetin, a carotenoid derivative (Fig. 1). *cis*-Crocetin and its glycosides are also present, however, they are the minor components in saffron. In addition, saffron also contains the flavonoid derivatives, picrocrocin and its aglycone safranal, in lower quantities. Picrocrocin is responsible for the bitter taste of saffron and has a potential value to be developed as a food additive [7,8]. Among the four crocins, crocin 1 is the most abundant in saffron and has been extensively studied for its pharmacological

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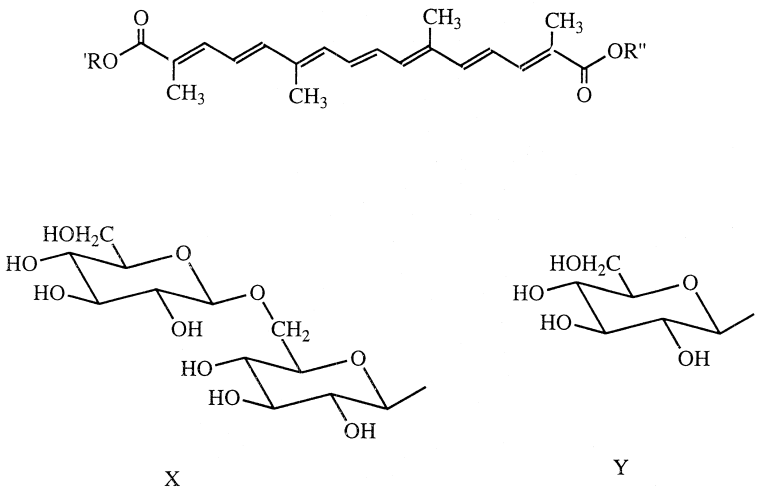

Crocin 1: R' = R" = X
Crocin 2: R' = X, R" = Y
Crocin 3: R' = X, R" = H
Crocin 4: R' = Y, R" = H
Crocetin: R' = R" = H

Fig. 1. Structures of crocetin and crocins 1–4.

effects [9–12]. It has been demonstrated that crocin derivatives, especially crocin 1, and the crude extract of saffron suppress tumor growth [5,13] and increase the diffusion of oxygen to the capillary endothelial cells [14,15]. Recently, the crude extract of saffron has been used clinically to relieve the symptoms of angina pectoris in China [16].

Quality control of saffron is therefore concentrated mainly on the determination of the major biologically active crocin analogues. Various analytical methods have been developed, including colorimetric measurement [17], thin-layer chromatography (TLC) scanning [18], high-performance liquid chromatography (HPLC) [19–23] and gas chromatography (GC) [23] assays. Previously published HPLC methods with either UV [19–21] or mass spectrometric (MS) [22] detection were generally used for separation and qualitative studies. For quantification, only one HPLC method has been reported. However, crocin analogues could not be quantified individually in their assay [23]. In the present work, a simple HPLC–UV method has been developed to simultaneously quantify the five major bioactive ingredients, crocins 1–4 and crocetin. The developed method was successfully applied to the quantification of these five components in three saffron samples and two Zhizi (*Gardenia jasminoides* Ellis), another crocin-containing TCM.

2. Experimental

2.1. Chemicals and materials

Two saffron (*Crocus sativus* L.) samples were obtained locally from Hong Kong herbal shops and had been imported from Spain and Tibet, respectively, and Iranian saffron was from the TCM Company in Nanjing, China. Two Zhizi (dried fruit of *Gardenia jasminoides* Ellis) samples were purchased from TCM shops in Hong Kong and Nanjing, respectively. 13-*cis*-Retinoic acid was purchased from Sigma (St. Louis, MO, USA). Crocetin and crocins 1–4 were isolated from the Spanish saffron [24]. The purity and structural identities of each

isolated compound were characterized and confirmed by HPLC, UV, IR, NMR and MS analyses.

2.2. Apparatus

The HPLC system consisted of a Hewlett-Packard (HP) 1100 chromatograph and a photo diode-array (PDA) multiple-wavelength UV detector. The column configuration consisted of a Nova-Pak C_{18} reserved-phase column (4 μm, 150×3.9 mm I.D.) and a HP C_{18} guard column (5 μm, 20×4 mm I.D.). UV absorption was measured either with a full spectrum (200–600 nm) or at 420 nm.

2.3. Chromatographic conditions

Gradient elution was employed using solvent systems A (methanol) and B (1% aqueous acetic acid, v/v) at ambient temperature. The gradient program used was as follows: initial 0–1 min, A–B (40:60, v/v); 1–6 min, linear change to A–B (55:45, v/v); 6–23 min, linear change to A–B (75:25, v/v); 23–25 min, linear change to A–B (90:10, v/v) and this was maintained for 5 min; and, at 30 min, there was a return to the initial conditions. The flow-rate was kept constant at 1.0 ml/min.

2.4. Calibration curves

Methanol stock solutions containing crocins 1–4 and crocetin were prepared and then diluted to appropriate concentration ranges for the construction of calibration curves. Each calibration curve was preformed in triplicate with five different concentrations (Table 1). The concentration of the internal standard, 13-*cis*-retinoic acid, was 500 μg/ml for all analyses. Calibration curves were constructed by spiking five analytes and the internal standard into the 80% methanol solution (5 ml) mixed with saffron (20 mg) prior to extraction. The resultant mixtures were extracted as described in Section 2.7. Aliquots (3 μl) of the extracts were analyzed by HPLC. For the control, extracts of saffron spiked with internal standard only were prepared in an identical manner and analyzed. Calibration curves were derived by plotting concentrations of each analyte as a function of peak area ratio differences (peak area $\text{ratio}_{\text{spiked}}$ − peak area $\text{ratio}_{\text{control}}$) between spiked and control extracts.

2.5. Accuracy and precision

The measurements of intra- and inter-day variability were utilized to determine the precision and accuracy of the developed assay. Known quantities of five analytes and internal standard were added to Spanish saffron (20 mg) prior to extraction. Control samples spiked with the internal standard only were also prepared similarly. The resultant samples were extracted and analyzed as described in Section 2.7. The peak area ratio difference between testing and control samples for each analyte was calculated, and the quantity of each analyte was subsequently obtained from the corresponding calibration curve. The relative standard deviation (RSD) was taken as a measure of precision and the percentage difference between amounts determined and spiked was considered as a measure of accuracy. The inter-day reproducibility was examined on three separate days.

2.6. Limits of detection

Aliquots of analytes were spiked into the 80% aqueous methanol solution (5 ml) mixed with Span-

Table 1
Calibration curves derived for crocins 1–4 and crocetin

Compound	Retention time (min)	$y=mx+c$		Correlation coefficient (r^2)	Concentration (μg/ml)
		Slope (m)	Intercept (c)		
Crocin 1	5.3	0.00431	0.0321	0.9991	30–300
Crocin 2	6.9	0.00375	−0.0436	0.9993	20–200
Crocin 3	12.0	0.00439	−0.0227	0.9990	10–100
Crocin 4	14.5	0.00173	−0.0030	0.9996	5–50
Crocetin	22.5	0.00291	0.0006	0.9988	5–50

ish saffron (20 mg) to provide concentrations in the range of 0.1 to 1.0 μg/ml. The samples were extracted and analyzed in a similar manner. The limit of detection for each analyte was determined when the ratio of the testing peak signal-to-noise ratio was greater than five.

2.7. Quantification of crocetin and crocins 1–4 in saffron and Zhizi

To ground dried saffron (20 mg) or Zhizi samples (100 mg), 5 ml of 80% methanol and the internal standard (500 μg/ml) were added. The methanol mixture was then stirred in the dark at ambient temperature for 2 h. The resultant mixture was filtered and aliquots (3 μl for saffron and 5 μl for Zhizi) of the filtrate were analyzed by HPLC. The contents of the analytes were determined from the corresponding calibration curves.

3. Results and discussion

Full PDA UV–visible spectra (200–600 nm) of saffron extract obtained from the PDA detector exhibited three absorption bands (data not shown). The first band, at 250–260 nm, had the lowest absorptivity and this was attributed to the glycoside bonds of both *cis*- and *trans*-carotenoids and picrocrocin. The second band, at 320–340 nm, was characteristic of all *cis*-carotenoids. The third band, at 400–500 nm, which had the highest absorptivity, corresponded to all *trans*-carotenoids. These data are in good agreement with the published results [21,22,25–27]. Therefore, *trans*-carotenoids can be selectively determined without interference with other components present in the saffron extract by choosing an appropriate wavelength range between 400 and 500 nm.

Blank controls are generally unavailable for the study of herbal materials and, thus, calibration is normally conducted by adding authentic samples to pure organic solutions without using any internal standard. Therefore, reproducibility and extraction yield become critical for quantification of the principal components in herbs. In the present study, this problem was solved using an internal standard. For the analysis of herbs, the internal standard was spiked prior to extraction, whereas for the construction of calibration curves, either authentic analytes and internal standard or the internal standard alone (as control) were added to the herbal powders prior to extraction. Extraction was conducted at room temperature in the dark to avoid possible hydrolysis and/or degradation, since saffron carotenoids are very sensitive to light and heat [21]. Calibration curves were then derived as a function of the concentration of each analyte versus the peak area ratio differences between spiked and non-spiked (control) herbal extracts. The quantification was therefore independent of variations in extraction recoveries for different analytes. As shown in Table 1, all five calibration curves exhibited good linear regression. The developed method resulted in good reproducibility, with overall intra- and inter-day variations of less than 6 and 11%, respectively (Table 2).

An appropriate internal standard is very important in the study. Various compounds that are structurally close to crocins and have a relatively strong UV absorption at 400–500 nm, including all-*trans*-retinoic acid, were tested. However, 13-*cis*-retinoic acid had the most appropriate retention time compared with the other compounds examined. It was well resolved with a baseline separation from all crocin analogues, and the analysis could be completed within 30 min (Fig. 2). Therefore, 13-*cis*-retinoic acid was a suitable internal standard. Furthermore, 13-*cis*-retinoic acid had a maximum absorption at 350–430 nm, while *trans*-crocins have maximum absorption at 400–500 nm. All analytes could be detected with relatively high sensitivities when absorption was measured at 420 nm, a wavelength within the overlapping maximum absorption ranges for both crocins and 13-*cis*-retinoic acid (Fig. 2). The results demonstrated that the developed HPLC method with UV detection at 420 nm showed good sensitivities for all analytes. Detection limits were 0.2 μg/ml (50 ng/mg of the dried herb) for crocins 1–3 and 1 μg/ml (250 ng/mg of the dried herb) for both crocin 4 and crocetin and all were far below the quantities present in herbs.

Under the chromatographic conditions used in this study, five compounds, namely crocins 1–4 and crocetin, were well-resolved with baseline separation. Typical chromatograms of the spiked standards

Table 2
Intra- and inter-day variability for the assay of crocins 1–4 and crocetin

Concentration (μg/ml)	Intra-day (n=3)			Inter-day (n=3)		
	Found	RSD (%)[a]	Accuracy (%)[b]	Found	RSD (%)[a]	Accuracy (%)[b]
Crocin 1						
90	96.29±5.46	5.7	7.0	98.14±5.72	5.8	9.0
250	253.36±1.45	0.6	1.3	252.28±1.95	0.8	0.9
Crocin 2						
80	80.62±0.62	0.8	0.8	79.38±0.94	1.2	0.8
180	179.82±0.62	0.3	0.1	180.00±0.46	0.3	0
Crocin 3						
40	40.55±1.49	3.7	1.4	43.36±4.56	10.5	8.4
90	96.73±2.87	3.0	7.5	96.13±5.13	5.3	6.8
Crocin 4						
15	15.61±0.67	4.3	4.1	15.80±0.33	2.1	5.3
40	40.46±0.33	0.8	1.2	40.85±0.58	1.4	2.1
Crocetin						
15	15.12±0.40	2.6	0.8	15.01±0.60	4.0	0.1
40	39.97±0.70	1.7	0.1	40.15±0.78	1.9	0.4

[a] RSD (%)=(SD/mean)×100.
[b] Accuracy (%)=[(mean concentration measured−concentration spiked)/concentration spiked]×100.

and extracts of saffron and Zhizi detected at 420 nm are shown in Fig. 2. Identification of the analytes in herbal extracts was confirmed by direct comparison of both UV spectra and the retention time of each analyte with those obtained from the authentic standards. In the chromatograms of herbal extracts (Fig. 2A and B), two minor peaks (7 and 8), corresponding to *cis*-crocins, were also observed. Due to their low absorption at 420 nm, they appeared with very low intensities. These two constituents were completely separated from the *trans*-crocins, thus, they did not affect the quantitative analyses.

The developed HPLC assay was subsequently applied to the simultaneous determination of the five major ingredients of saffron in three available saffron and two Zhizi samples. Representative chromatograms of the extracts of saffron and Zhizi are shown in Fig. 2. As shown in Table 3, all five compounds were found in both Spanish and Iranian saffron samples, whereas crocetin and crocin 4 could not be determined in Tibetan saffron and two Zhizi samples. The amounts of crocins, especially crocin 1, present in the Spanish saffron are significantly higher than those in the other two saffron samples tested. Furthermore, the overall quantity of crocins present in saffron is markedly higher than that in Zhizi samples. However, Zhizi, a much cheaper TCM derived from the dried fruit of *Gardenia jasminoides* Ellis [28], may also be used as an alternative source for the isolation of crocin analogues.

In conclusion, a novel, simple and specific HPLC method has been developed to simultaneously quantify crocins 1–4 and crocetin, five major biologically active constituents, in saffron. This assay is sensitive and reproducible, and has been fully validated. It was successfully applied to the quantification of these five compounds in three saffron and two Zhizi samples. Results indicate that the developed HPLC assay can be readily utilized as a quality control method for crocin-containing natural products.

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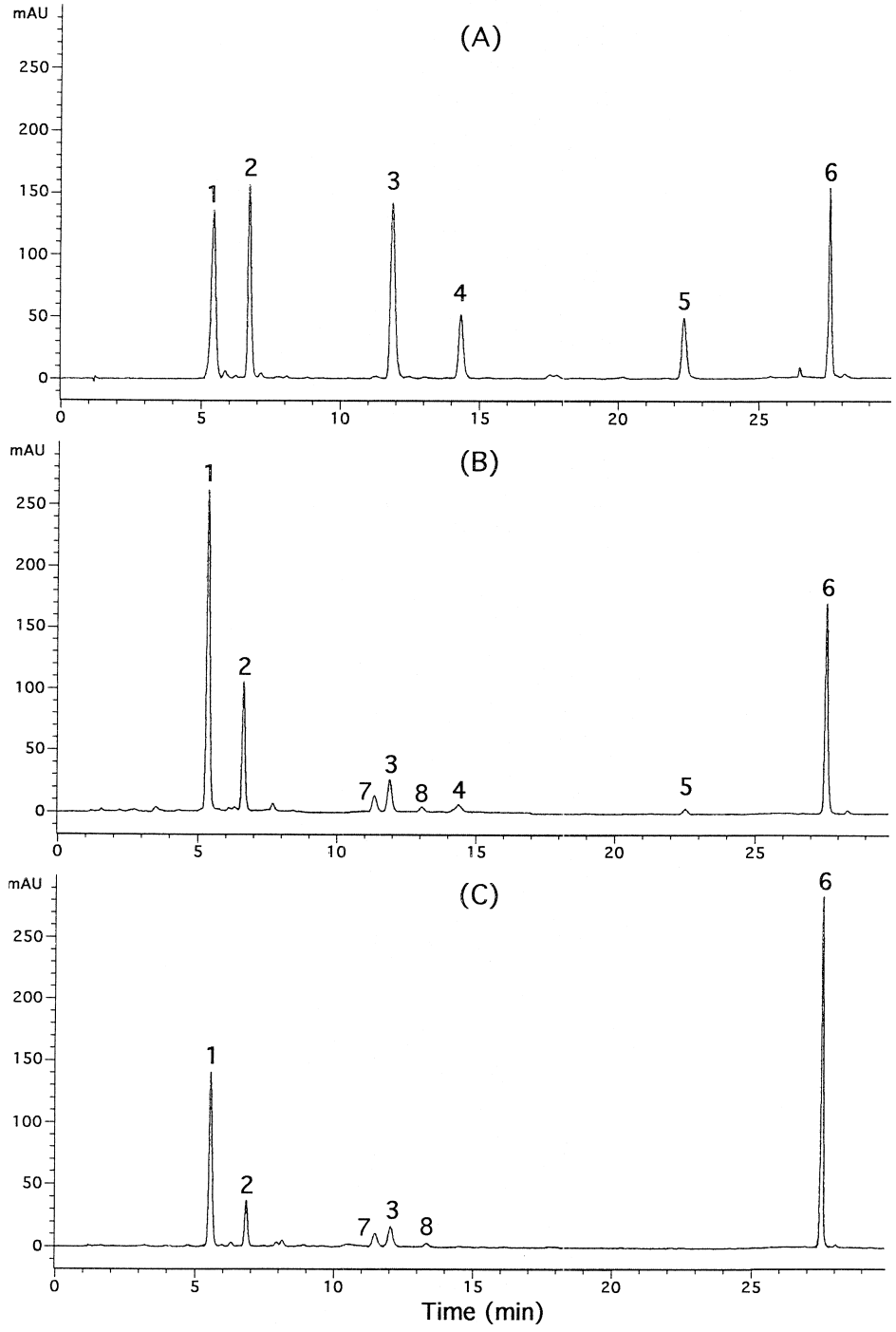

Fig. 2. Representative HPLC chromatograms of the extracts of (A) Spanish saffron spiked with standards, (B) Iranian saffron and (C) Zhizi purchased from Nanjing. Peaks: 1=crocin 1, 2=crocin 2, 3=crocin 3, 4=crocin 4, 5=crocetin, 6=13-*cis*-retinoic acid (internal standard), 7 and 8, *cis*-crocins.

Table 3
Quantities of crocins 1–4 and crocetin in herbs obtained from different sources

Compound	Saffron (%)[a]			Zhizi (%)[a]	
	Spain	Iran	Tibet	Hong Kong	Nanjing
Crocin 1	9.00±0.71	2.90±0.21	2.80±0.06	0.20±0.01	0.37±0.02
Crocin 2	4.60±0.35	1.90±0.12	1.70±0.06	0.06±0.003	0.15±0.004
Crocin 3	1.30±0.12	0.87±0.05	0.71±0.05	0.05±0.003	0.09±0.01
Crocin 4	0.36±0.06	0.23±0.01	nd	nd	nd
Crocetin	0.06±0.01	0.05±0.01	nd	nd	nd

[a] Data are presented as the percentage of the dried herbs, and expressed as mean±SD of triplicate analyses for each herb.
nd=not detected.

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References

[1] S.C. Nair, B. Panikkar, K.R. Panikkar, Cancer Lett. 57 (1991) 109.
[2] J.V. Bhat, R. Broker, Nature 172 (1953) 544.
[3] S.C. Nair, S.K. Kurumboor, J.H. Hasegawa, Cancer Biother. 10 (1995) 257.
[4] D. Basker, M. Negbi, Econ. Bot. 37 (1983) 228.
[5] P.A. Tarantilis, H. Morjani, M. Polissiou, M. Manfait, Anticancer Res. 14 (1994) 1913.
[6] X.B. Ni, Chinese Trad. Herbal Drugs 23 (1992) 100.
[7] J.L. Iborra, M.R. Castellar, M. Cánovas, A. Manjón, Biotechnol. Lett. 14 (1992) 475.
[8] H. Pfander, H. Schurtenberger, Phytochemistry 20 (1982) 1039.
[9] M.S. Zarghami, D.E. Heinz, Phytochemistry 10 (1972) 2755.
[10] H. Himeno, K. Sano, Agric. Biol. Chem. 51 (1987) 2395.
[11] J.L. Iborra, M.R. Castellar, M. Cánovas, A. Manjón, J. Food Sci. 57 (1992) 714.
[12] H. Pfander, F. Wittwer, Helv. Chim. Acta 58 (1975) 1608.
[13] M.J. Salomi, S.C. Nair, L.R. Panikker, Nutrit. Cancer 16 (1991) 67.
[14] J.V. Gainer, G.R. Nugent, J. Neurosurg. 45 (1976) 535.
[15] M.G. Chisolm, J.L. Gainer, G.E. Stoner, J.V. Gainer, Atherosclerosis 15 (1972) 327.
[16] Y.L. Bai, M.L. Ke, J.D. Liu, Res. Trad. Chin. Med. 5 (1991) 33.
[17] Y. Kimura, Y. Nishikawa, Yakugaku Zasshi 73 (1953) 25.
[18] R.L. Jin, Z.P. Qiao, S.D. Zhou, Y.L. Ye, J.X. Zhou, J. Nanjing Coll. Pharm. 17 (1986) 247.
[19] H. Pfander, M. Rychener, J. Chromatogr. 234 (1982) 443.
[20] M.R. Castellar, H. Montijano, A. Manjón, J.L. Iborra, J. Chromatogr. 648 (1993) 187.
[21] P.A. Tarantilis, M. Polissiou, M. Manfait, J. Chromatogr. A 664 (1994) 55.
[22] P.A. Tarantilis, G. Tsoupras, M. Polissiou, J. Chromatogr. A 699 (1995) 107.
[23] V. Sujata, G.A. Ravishankar, L.V. Venkartaraman, J. Chromatogr. 624 (1992) 497.
[24] V.K. Dhingra, T.R. Seshadri, Ind. J. Chem. 13 (1975) 339.
[25] G. Speranza, G. Dada, P. Manitto, D. Monti, P. Grammatica, Gazz. Chim. Ital. 114 (1984) 189.
[26] D. Basker, M. Negbi, J. Assoc. Pub. Anal. 3 (1985) 65.
[27] H. Himeno, K. Sano, Agric. Biol. Chem. 51 (1987) 2395.
[28] X. Liu, Z.Y. Qia, J. Fu, J. China Pharmaceutical University 27 (1996) 734.