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Methods for the analysis of the saffron metabolites crocin, crocetins, picrocrocin and safranal for the determination of the quality of the spice using thin-layer chromatography, high-performance liquid chromatography and gas chromatography

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ABSTRACT

Saffron spice is the dry stigmata of *Crocus sativus* L. Methods have been developed for the determination of the quality of the spice using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). TLC and HPLC gave comparable results for crocin and crocetins (colour principles), picrocrocin (bitter substance) and safranal (flavour). Similarly, the determination of safranal by GC was in agreement with analysis by TLC and HPLC. Separation of the constituents was achieved by silica gel G TLC using an *n*-butanol–acetic acid–water (4:1:1) system. The resolution of crocin, crocetins and picrocrocin by HPLC was obtained using a Shimadzu 15-cm CLC-ODS column with 20–80% acetonitrile in water as the eluent; for safranal an isocratic run with 76% acetonitrile in water was suitable. GC was adopted only for the determination of safranal using a Shimadzu 5% SE-30 column. HPLC was most suitable for the detection of adulterants and was simpler and more efficient for quality analysis. The TLC method was time-consuming and also gave an overestimation of the colour principles.

INTRODUCTION

Commercially used saffron is the dried stigmata of *Crocus sativus* L. It is popular because of its delicate aroma and attractive colour and is used as a food additive [1]. Saffron is cultivated in countries such as Spain, Italy, Iran, Switzerland and India. In India, its cultivation is restricted to the Himalayan state of Jammu and Kashmir. *Crocus sativus* plants require strict agroclimatic conditions for their growth, which influence the quality of the spice. The harvesting of the stigmata is a labour-intensive process and requires processing of 150 000 flowers for 1 kg of spice. This spice costs around $2000/kg in the international market.

Samples obtained from different geographical locations and from different processing methods are expected to show variations in quality with respect to colour (crocin and crocetins: carotenoid derivatives), flavour (safranal: a monoterpene) and the bitter principle (picrocrocin: a monoterpene glucoside). Moreover, due to the high cost of saffron, adulteration is rampant in India and the international market. The determination of the quality of saffron is an important consideration for the spice industry and for consumers. Of the various analytical methods described, the ISO recommended a thin-layer chromatographic (TLC) method for qualitative analysis of saffron [2]. However, this method is inadequate for grading saffron with respect to its vital constituents. The qualitative methods adopted for the determination of the colour and flavour profiles have been reviewed by Sampathu *et al.* [3]. The colour component is determined using a 1.5% potassium dichromate solution as a reference [4]. A determination method for flavour has been developed using gas chromatography (GC) [5] and spectrophotometry [6]. The determination of crocin,

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picrocrocin and safranal has been carried out by high-performance liquid chromatography (HPLC) [7,8]. No other methods have been published for the determination of these constituents.

The use of analytical methods for grading saffron has not been developed. In this work attempts have been made to develop sensitive methods for grading and assessing purity.

EXPERIMENTAL

Standards

An authentic sample of safranal was obtained from Malti-chem Research Centre (Baroda, India). An extract from the fruits of *Gardenia jasminoides* [9], collected from Wayanad District, Kerala, India, was used as the standard for crocin. Authentic crocetin was obtained from Sigma (St. Louis, MO, USA) (Cat. No. C 3398). The authenticity of the crocin standard was confirmed by the formation of a violet colour on the addition of concentrated H_2SO_4 and a green colour on the addition of concentrated HNO_3 [3]. All the reagents used were from SD Fine Chemicals (Boisar, India) unless stated otherwise.

A picrocrocin spot of R_F 0.32 was obtained by TLC [2] of the authentic saffron extract [in 80% (v/v) ethanol] using *n*-butanol–acetic acid–water (4:1:1). Picrocrocin was identified under UV light. The picrocrocin spot was eluted in 80% ethanol and concentrated to a known volume.

Source of saffron and extraction procedures

Saffron stigmata were obtained from fields at Pampore valley, Kashmir, India. Saffron samples were obtained from the local market. A 100-mg mass of dry stigmata was extracted with 5 ml of cold 80% (v/v) ethanol in a pestle and mortar, centrifuged at 5000 *g* for 10 min and washed twice with 5 ml each of the same solvent. The supernatant was used for further analysis by spectrophotometric and TLC procedures.

Thin-layer chromatography

Plates impregnated with approximately 0.25-mm-thick silica gel G were used for all the analyses. The plates were activated before use by heating at 100°C for 1 h. An aliquot of the alcoholic supernatant (0.1 ml) was spotted onto the plates. The developing chamber was saturated with the solvent *n*-butanol–acetic acid–water (4:1:1) for 6 h before running at room temperature in the dark. Crocin and crocetins were visible in white light whereas picrocrocin was detected under a UV source (254 nm) as a dark brown fluorescing spot.

For the determination of safranal, 2 ml of the 80% (v/v) ethanol extract were diluted to 4 ml with distilled water and separated five times with 10 ml of diethyl ether. The ether layers were pooled, air-dried and the volume made up to 0.1 ml with diethyl ether. This extract was spotted onto TLC plates which were developed in a hexane–ethyl acetate (9:1) solvent system under the described conditions. The safranal spot was visualized as a dark reddish brown spot on spraying with 2,4-dinitrophenylhydrazine (DNPH) reagent. The reagent was prepared by dissolving 0.5 g of DNPH [Glaxo Lab. (I), Bombay, India] in 25 ml of methanol acidified with one drop of concentrated H_2SO_4 [10].

After the spots had been identified, they were eluted on a preparatory scale into known volumes of 80% (v/v) ethanol and analysed spectrophotometrically at their λ_{max} (specified below).

Spectrophotometry

The supernatant (1 ml) was diluted to 5 ml with 80% (v/v) ethanol for analysis using a Shimadzu (Tokyo, Japan) UV-160 A, UV–visible recording spectrophotometer. The absorption maxima and extinction coefficients for crocin and picrocrocin were 443 nm, 89 000 and 250.5 nm, 10 100, respectively [7]; λ_{max} for safranal was 308 nm through a 1-cm pathlength as reported previously [7]. A standard graph was prepared by measuring the absorption of safranal at 308 nm.

Sample preparation and HPLC conditions

The extract of authentic stigmata in 80% (v/v) ethanol was passed through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA) and eluted with 100% acetonitrile. The eluent was flash-evaporated to dryness using a RotaVapor (Buchi, Switzerland) and redissolved in 20% (v/v) acetonitrile in water before injection onto the HPLC column. This step was necessary to clean the extract and was more efficient than passing the samples through a 0.22-μm Millipore filter (Millipore, Bedford, MA, USA).

A Shimadzu HPLC LC-6A system integrated with a Shimadzu SCL-6A system controller and a

SPD-6AV UV–visible spectrophotometric detector was used for the analysis of all the compounds. A Shimadzu 15 cm × 4.9 mm I.D. CLC-ODS column with a $5 \cdot 10^{-8}$ cm pore size and $10 \cdot 10^{-8}$ cm particle size was used for the separation and identification of the compounds. A Rheodyne Shimadzu Model 7125 injector was used to inject 10 μl of the sample from a 20-μl Hamilton (Hamilton, Reno, NV, USA) straight-edge needle syringe onto the column. All data were recorded on a Shimadzu CR4A recording system.

Crocin, crocetins and picrocrocin were separated on a gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min at a flow-rate of 0.5 ml/min. Picrocrocin was detected at 250.5 nm and the colour principles at 443 nm.

The separation of safranal was attempted using the following two methods: (i) a gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min at a flow-rate of 0.5 ml/min, detection at 308 nm; (ii) an isocratic run with 76% (v/v) acetonitrile in water at a flow-rate of 0.5 ml/min, detection at 308 nm.

All solvents used were of HPLC grade and filtered through a 0.45-μm filter (Millipore). A standard graph of area *versus* concentration was prepared using the standards.

Gas chromatography of safranal

The ether extract of safranal, as prepared for the TLC procedure, was used for the analysis. A 10-μl capacity Hamilton beveled syringe was used to inject 1 μl of the sample.

A Shimadzu 5% SE-30, 3-m stainless-steel column was fitted on a Shimadzu GC-15A gas chromatograph with an online flame ionization detector. An isothermal run at 150°C with nitrogen as the carrier gas at a flow-rate of 30 ml/min was used. All data were recorded on a Shimadzu CR4A recorder.

Statistical analysis of the samples analysed by TLC, HPLC and GC were carried out using Duncan's multiple range test and Student's *t*-test [11,12].

RESULTS AND DISCUSSION

Thin-layer chromatography

Crocin, picrocrocin and crocetins could be resolved on TLC plates run in the solvent system *n*-butanol–acetic acid–water (4:1:1) (Table I). For quantitative purposes the four crocetins, identified at R_F values of 0, 0.41, 0.75 and 0.98 were considered as total crocetins. The identification of individual crocetins was not carried out. Safranal, however, remained at the origin when run in this solvent system (Table I). As it interfered with the resolution of one of the crocetin derivatives, another solvent system (hexane–ethyl acetate, 9:1) was tested. In this system, safranal was well resolved (Table I). Moreover, another extraction procedure had to be suitably adopted (as detailed under Experimental) to resolve safranal using hexane–ethyl acetate (9:1).

TLC spots corresponding to crocin, crocetins, picrocrocin and safranal were eluted in 80% (v/v) ethanol and determined spectrophotometrically at their λ_{max} (see under Experimental). An average value of five replicates is given in Table II.

Direct spectrophotometric determination of the crude extract [*i.e.* the stigmata extract in 80% (v/v) ethanol] at various λ_{max} values corresponding to crocin, crocetins, picrocrocin and safranal did not

TABLE I

TLC ANALYSIS OF SAFFRON METABOLITES

TLC plates impregnated with 0.25-mm-thick Silica gel C were run with various solvent systems.

Component	R_F		Mode of detection
	n-Butanol–acetic acid–water (4:1:1)	Hexane–ethyl acetate (9:1)	
Crocin	0.63	–	Visible light
Picrocrocin	0.32	–	UV light
Crocetins	0.98, 0.75, 0.41, 0	–	Visible light
Safranal	0	0.78	DNPH reagent

TABLE II

DETERMINATION OF IMPORTANT CONSTITUENTS OF SAFFRON STIGMATA USING DIFFERENT ANALYTICAL PROCEDURES

TLC: conditions as in Table I. Crocin, crocetins and picrocrocin were eluted in 80% (v/v) ethanol and safranal in diethyl ether. Eluents were determined spectrophotometrically at a λ_{max} of 443, 250.5 and 308 nm, respectively. HPLC: column CLC-ODS (150 mm × 4.9 mm I.D.) using a gradient run from 20 to 80% (v/v) acetonitrile in 20 min for crocin, crocetins and picrocin. For safranal an isocratic run at 76% (v/v) acetonitrile was used. The flow-rate in both instances was 0.5 ml/min. The retention times under these conditions were: crocin: 13.5 min; crocetins 14–18 min; picrocrocin, 8 min and safranal 6 min. GC: safranal was separated on a 5% SE-30 3-m stainless-steel column using nitrogen as the carrier gas at a 30 ml/min flow-rate and detected using a flame ionization detection system. Values are mean ± S.D.

Method	Crocin (mg%)	Crocetins (mg%)	Picrocrocin (mg%)	Safranal (mg%)		
TLC	5.54 ±0.16	2.20 ±0.16	10.18 ±0.4	0.80 ± 0.02 (*A*)		
HPLC	5.86 ±0.15	2.15 ±0.064	11.23 ±0.23	0.83 ± 0.008 (*B*)		
GC[a]	–	–	–	0.81 ± 0.013 (*C*)		
Student's *t*-test[b]	3.16*	0.643 (NS)	4.4**	3.39** (*A–B*)	1.2 (NS) (*A–C*)	2.55* (*B–C*)

[a] Analysis of crocin, crocetins and picrocrocin by GC was not carried out.
[b] NS = Not significant; * = significant at $p \leq 0.05$; ** = significant at $p \leq 0.01$.

yield reproducible results. Moreover, it resulted in an underestimation of the constituents and hence was not suitable for analysis. In contrast, TLC followed by spectrophotometry gave reproducible results.

High-performance liquid chromatography

A gradient run from 20 to 80% (v/v) acetonitrile was suitable for the resolution of crocin, crocetins and picrocrocin (Figs. 1 and 2). The retention times for these compounds were 13.5, 14–18 and 8 min, respectively. The solvents used in this study were similar to those reported by Himeno and Sano [7] who used a Hiber LiChrosorb RP-18 (particle size 5 μm, column size 250 mm × 4 mm I.D.) (Cica-Merck) column which resolved crocin and picrocrocin, whereas the resolution of safranal was not as distinct as the former two compounds. In the present study the experiment with a gradient run could not resolve safranal (Fig. 3). However, an isocratic run (Fig. 4) using 76% acetonitrile gave a distinct peak of safranal at 6 min retention time. The resolution of safranal obtained in the isocratic run using 76% acetonitrile was far superior and can be used for routine analyses.

Crocetins were determined by summing the peak areas of different crocetin peaks (Fig. 1). The

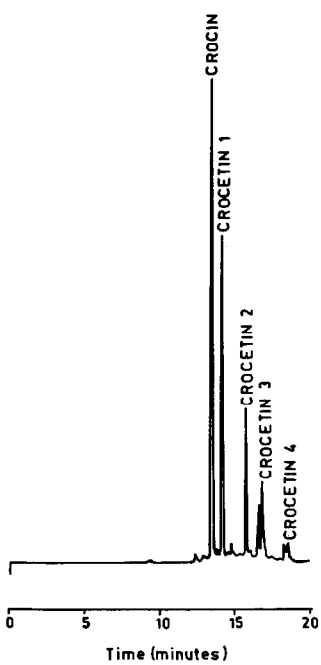

Fig. 1. HPLC profile of crocin and crocetins. Solvent, gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 × 4.9 mm I.D.); detection wavelength, 443 nm.

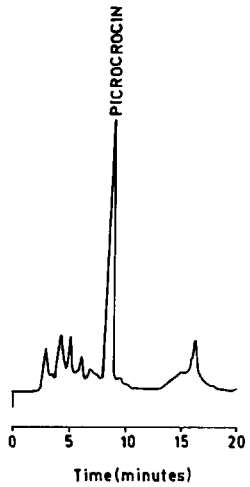

Fig. 2. HPLC profile of picrocrocin. Solvent, gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 mm × 4.9 mm I.D.); detection wavelength, 250.5 nm.

concentrations of crocin, crocetin, picrocrocin and safranal obtained by the conditions standardized in this study are shown in Table II.

Gas chromatography

SE-30 and Carbowax columns were used with isothermal and thermal gradient programmes. It was found that an isothermal run at 150°C on an SE-30 column resolved safranal into a sharp single peak at a retention time of 3.6 min (Fig. 5). A quantitative determination of safranal was achieved using this method and the results were compared with those obtained by TLC and HPLC (Table II). GC determinations of the colour and bitter principles were not carried out as this required the derivatization of the compounds.

Statistical analysis of the three methods used showed that the results obtained by the HPLC method were significantly different from those of the TLC and GC methods (Table II), whereas the results

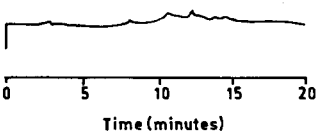

Fig. 3. HPLC profile of safranal (method i). Solvent, gradient run from 20 to 80% (v/v) acetonitrile, in water in 20 min; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 × 4.9 mm I.D.); detection wavelength, 308 nm.

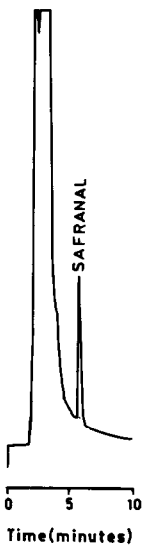

Fig. 4. HPLC profile of safranal (method ii). Solvent, 76% (v/v) acetonitrile in water; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 mm × 4.9 mm I.D.); detection wavelength, 308 nm.

of TLC and GC were comparable. In general, the concentrations of all the metabolites determined by HPLC were higher than those determined by TLC and GC.

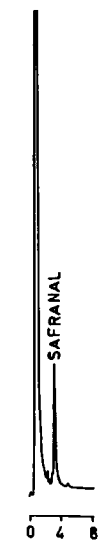

Fig. 5. GC profile of safranal. Temperature, 150°C; carrier gas, nitrogen (30 ml/min); column, Shimadzu SE-30 5% 3-m stainless steel; detector, flame ionization.

TABLE III

ANALYSIS OF MARKET SAMPLES OF SAFFRON

TLC and HPLC conditions as in Table II. Values are mean ± S.D.

Method	Crocin (mg%)	Crocetins (mg%)	Safranal (mg%)	Picrocrocin (mg%)
TLC	6.73 ± 0.12	0.81 ± 0.16	0.46 ± 0.01	0.48 ± 0.021
HPLC	4.8 ± 0.09	0.92 ± 0.14	0.4 ± 0.012	0.32 ± 0.02

Detection of adulteration

For the detection of adulteration in saffron, TLC and HPLC methods were tested (Table III). In many samples analysed by TLC there was overlapping of the adulterant spot and crocin, thereby rendering this method unsuitable for analysis, whereas the same sample analysed by the HPLC method gave a broad peak which was distinct from the crocin peak of the authentic sample (Fig. 6). Hence it was found that HPLC was most suitable method for the detection of adulterants in saffron.

CONCLUSIONS

TLC methods followed by spectrophotometry for the determination of crocin, crocetins, picrocrocin and safranal were reproducible. The GC method for the analysis of safranal gave comparable results to those obtained by TLC followed by spectrophotometry, whereas the HPLC method was sensitive and simpler as only one method of sample preparation and one set of solvents were required. The HPLC method was also the most suitable for the detection of adulterants.

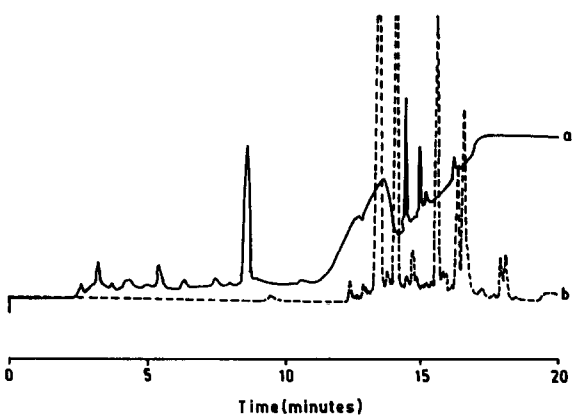

Fig. 6. HPLC analysis of saffron market sample. Solvent, gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 mm × 4.9 mm I.D.); detection wavelength, 443 nm. (a) Market sample; (b) stigmata from field.

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