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Short communication

Quantitative high-performance liquid chromatographic method to analyse commercial saffron (*Crocus sativus* L.) products.

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Abstract

An HPLC method to check components and purity in commercial saffron by photodiode array detection has been developed. The method was suitable for the standard analysis of commercial saffron. Therefore, 10 saffron metabolites responsible for the taste, flavour and colour were identified and quantified with high selectivity, precision and accuracy. Also, some artificial colorants, which can be used as adulterants, were also detected and identified. Three different saffron types were studied and their metabolite concentrations determined at different wavelengths.

Keywords: Saffron; Picrocrocin; Crocin; Safranal

1. 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 [1,2], as well as for its therapeutic properties (e.g., antitumoral activity) [3–5]. Spain is the main saffron producer, where products are classified by their quality in three grades: Mancha, Río and Sierra [6]. Due to its high value, saffron is susceptible to adulteration, usually by adding other plant materials dyed with artificial colorants (e.g., tartracine) to produce an increase in weight and yellow colour [1,2].

The saffron-coloured compounds are crocins, a family of unusual water-soluble carotenoid mono- and di-glycosyl esters of a polyene dicarboxylic acid, named crocetin, where D-glucose and D-gentobiose occur as carbohydrate residues. The digentiobiosyl ester of crocetin, called α-crocin, is the major component [7,8]. Picrocrocin is the main substance responsible of the bitter taste in saffron, it is a colourless glycoside, the sugar moiety of which is D-glucose, and 2,6,6-trimethyl-4-hydroxy-1-carboxaldehyde-1-cyclohexene (HTCC) the aglycone. Safranal is the volatile oil responsible of the characteristic saffron odour and aroma. This compound is obtained from picrocrocin and HTCC during the saffron drying process [1,2,6,9,10], as reported in Fig. 1.

Methods for detection and quantification of saffron metabolites are not unified. For saffron quality and stability studies, UV–Vis spectrometric methods are used [11–16]. The absorbance measurements at 250

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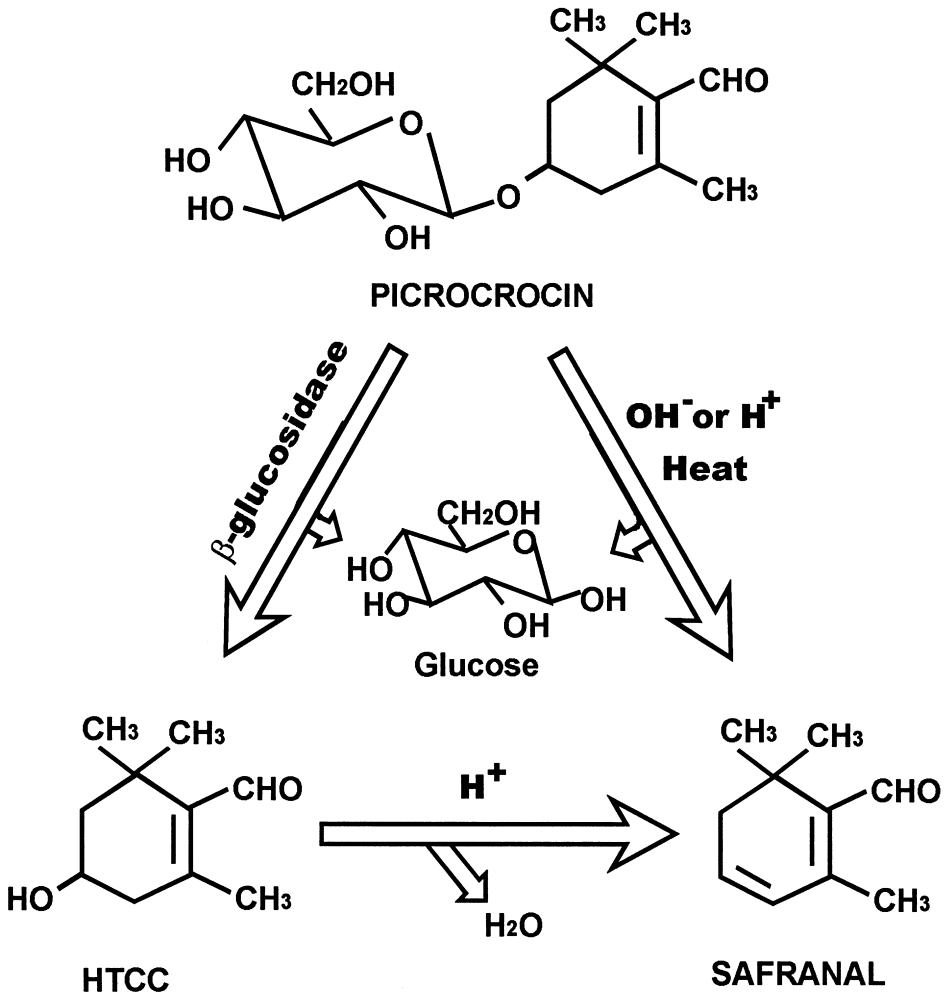

Fig. 1. Scheme of the chemical and enzymatic conversion of picrocrocin to HTCC and safranal.

nm (λ_{max} of both picrocrocin and HTCC), 310 nm (λ_{max} of safranal) and 440 nm (λ_{max} of crocins), are considered to be appropriate for identifying the taste, flavour and colour, respectively, of this spice. However, the identification and quantification of each compound in the saffron extract cannot be made by these methods. Several analytical and preparative methods of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have been developed to analyse saffron [7,17–24]. They have focused on the separation, the identification and/or the purification of saffron components. However, a quantitative method for saffron analysis, to correlate the saffron constituents with the quality of the commercial spice has not been reported. The aim of this paper was to develop an easy HPLC method for saffron analysis, which permitted simultaneous detection, identification and quantification of each of the secondary metabolites in commercial saffron samples, and some possible saffron adulterants.

2. Experimental

2.1. Materials

Three saffron types (Mancha, Sierra and Río), a

Mancha commercial saffron sample, and plant adulterants (*Cuscuta planiflora*, ligules of *Calendula officinalis* and stigmas of *Zea mays*) were obtained from local suppliers. Tartracin and methyl orange (Aldrich) and Ponceau S (Sigma) were used as colour adulterants. 4-Nitroaniline (Fluka) was used as internal standard. Methanol (Merck) was of chromatographic purity. Water was double distilled and purified through a Millipore system (Milli-Q).

2.2. Equipment

HPLC analyses were performed on a Shimadzu HPLC system equipped with two LC-6A pumps, a UV–Vis SPD-M6A photodiode array detector, a SIL 9A automatic injector and a Nova-Pack RP C_{18} column (Waters, 15 cm length, 3.9 mm internal diameter, 4 μm particle size and 6 nm pore size).

2.3. Extraction of saffron metabolites

The extraction of metabolites was optimised as a function of the solvent, temperature, presence of light and stirring time, by measurement of the optical density of samples at 250, 310 and 440 nm, using a GBC UV–Vis Model 918 spectrophotometer. In all cases, 400 mg of saffron were ground and suspended in 200 ml of solvent, and magnetically stirred with light, or in darkness, at different temperatures (25, 30, 40 and 50°C). At regular time intervals, aliquots of 75 μl were taken, diluted with 2.925 ml of the assayed solvent to measure absorbance. A solution of methanol–water (50%, v/v) was selected as the best solvent, with magnetic stirring during 1 h in darkness at 25°C as optimal conditions to obtain all the saffron components, according to other authors [8,15,19,23]. Therefore, the sample preparation for HPLC analysis was performed at 2 mg/ml saffron concentration. After extracting, samples were centrifuged at 30 000 *g* during 20 min and filtered through a Whatman filter (GF/F, 0.7 μm) to separate the plant residue, which was discarded. Before HPLC analysis, 100 ml of 0.2 mg/ml 4-nitroaniline was added to 100 ml saffron extract. A 1 mg/ml standard saffron sample was thus obtained. The saffron extract concentration was modified depending on the assay as reported in the text.

To optimise the mobile phase and to determine the specificity of the method, the artificial colorants tartracin, methyl orange and ponceau S were added to the standard saffron samples (0.02 mg/ml final concentration) to simulate the presence of impurities and/or adulterants. Besides, extracts from *Cuscuta planiflora*, ligules of *Calendula officinalis* and stigmas of *Zea mays* were obtained and analysed under the same conditions of saffron samples. All sample preparations were performed into gauged flask.

2.4. Chromatographic method

A linear gradient elution of methanol (20–70%) at 1% methanol/min gradient speed and 1 ml/min flow-rate was selected as mobile phase, as a function of the good peak resolution, the shortest analysis time and the lowest solvent cost. All analyses were performed at least three times at 30°C. Samples of 50 μl were injected, and the saffron components and the artificial colorants added were detected and identified. Picrocrocin, HTCC and 3-gentiobiosil-kaempferol were determined at 250 nm, safranal at 310 nm, and all of the crocins, tartracin, Ponceau S and methyl orange at 440 nm, while the internal standard was detected at the three wavelengths. The chromatographic parameters were calculated according to Ref. [25]. The t_0 value for k' determinations was obtained by the eluent front retention time.

2.5. Quantitative determination

This was performed by the internal standard method, using 4-nitroaniline as internal standard. This compound was chosen by its separation and resolution from close peaks and by its detection at all assayed wavelengths. As the availability of pure standards of all saffron components is usually difficult, quantitative determinations were made as the ratios between each compound integration area and the internal standard integration area ($A_{\mathrm{Comp}}/A_{\mathrm{I.S.}}$), obtained at the wavelength of maximum absorbance of the respective compound.

2.6. Calibration data

Three series of saffron extracts (six samples each, at 0.25, 0.50, 0.75, 1.00 1.25 and 1.50 mg/l, respectively, where each sample was analysed six

times) were injected three times on three different days. Three calibration rates relating area ratio ($A_{Comp}/A_{I.S.}$) with saffron concentration (mg/ml) were obtained for each compound at their respective detection wavelength values. The final calibration straight lines for each compound were taken as the mean of the slopes, intercept and correlation values of the three straight lines obtained, according to Ref. [26]. A linearity range from 0.25 to 1.50 mg/ml saffron extract was observed for the 10 compounds analysed, obtaining slope values from 0.06 to 4.3 $(A_{Comp}/A_{I.S.})$/(mg/ml). Additionally, the R^2 range obtained was from 0.9874 to 0.9968, with the slope R.S.D. values lower than 1.5%, which indicated a high accuracy for the method.

3. Results and discussion

Fig. 2 shows the reversed-phase HPLC separation of metabolites from Mancha, Rio and Sierra saffron samples. Each chromatogram is depicted at three wavelengths (250, 310 and 440 nm), corresponding to the maximal height of each peak, and using the same optical density scale. Ten different secondary metabolites were identified in the saffron extract, whereas all of them were only present in the Mancha type saffron. The peaks were identified by their respective absorption spectra, obtained with the photodiode array detector at the maximum absorbance of each compound. The purity index values obtained were higher than 0.99 for all peaks [23].

Table 1 reports the chromatographic parameters of all identified peaks for the Mancha saffron sample (chromatogram in Fig. 2A). The number of detected metabolites, and the peak resolution values obtained by this chromatographic method, were better than those previously reported for both isocratic [8] and fast gradient systems [9,17–19,24]. Additionally, the method selectivity was also tested by the addition of several adulterants (tartracin, ponceau S and methyl orange) to the saffron sample. These adulterants were detected and identified, specifically and separately from the saffron metabolites without interference, by the respective absorption spectrum, with a high method selectivity. As can be seen, tartracin and ponceau S showed A_s values out of the accepted range (0.9–1.1, [25]), probably due to their low retention times. However, they were clearly separated and integrated. Methyl orange and the 10 saffron secondary metabolites gave acceptable A_s values. The longer retained compounds showed higher than recommended k' values (≈10, [25]), because it was necessary to prolong the operation time to separate all the compounds contained in the sample. Additionally, for all peaks, the R_s values were higher than the minimum accepted (1.5, [25]). On the other hand, samples of several plant adulterants (*Cuscuta planiflora*, ligules of *Calendula officinalis* and stigmas of *Zea mays*) [2,6] were also studied. No coloured compounds were extracted from these raw materials. Thus, they should act only as weight adulterants or as carriers to retain artificial colorants. Therefore, the performed chromatographic method conducted a good separation and resolution of the sample components, better than other proposed gradient conditions previously reported [7,8,18,19,23,24].

On the other hand, several quantitative parameters were also studied. Picrocrocin was used as standard to analyse the effect of saffron sample concentration, because this compound showed the least variability when several assays were performed. Six saffron concentrations (0.25–1.50 mg/ml) were analysed six times, and the obtained R.S.D. values were less than 2% of the 0.75–1.25 mg/ml saffron concentration range. Thus, 1 mg/ml was selected as standard saffron concentration for the injection sample. The method accuracy was shown by the low R.S.D. of the three straight line slopes (lower than 1.5%) obtained for each compound, as reported in Section 2. In addition, saffron samples stored at 4°C during 7 days did not show any chromatographic or spectroscopic differences with fresh samples.

Table 2 reports the quantitative analysis of the standard saffron types (Mancha, Sierra and Rio), depicted in Fig. 2, and other additional commercial Mancha types. As can be seen, the standard Mancha type showed the highest concentration for all secondary metabolites followed by the Rio and Sierra types. However, the commercial Mancha type showed, in general, a lower amount of saffron metabolites than the standard Mancha type. It showed mainly lower picrocrocin concentrations,

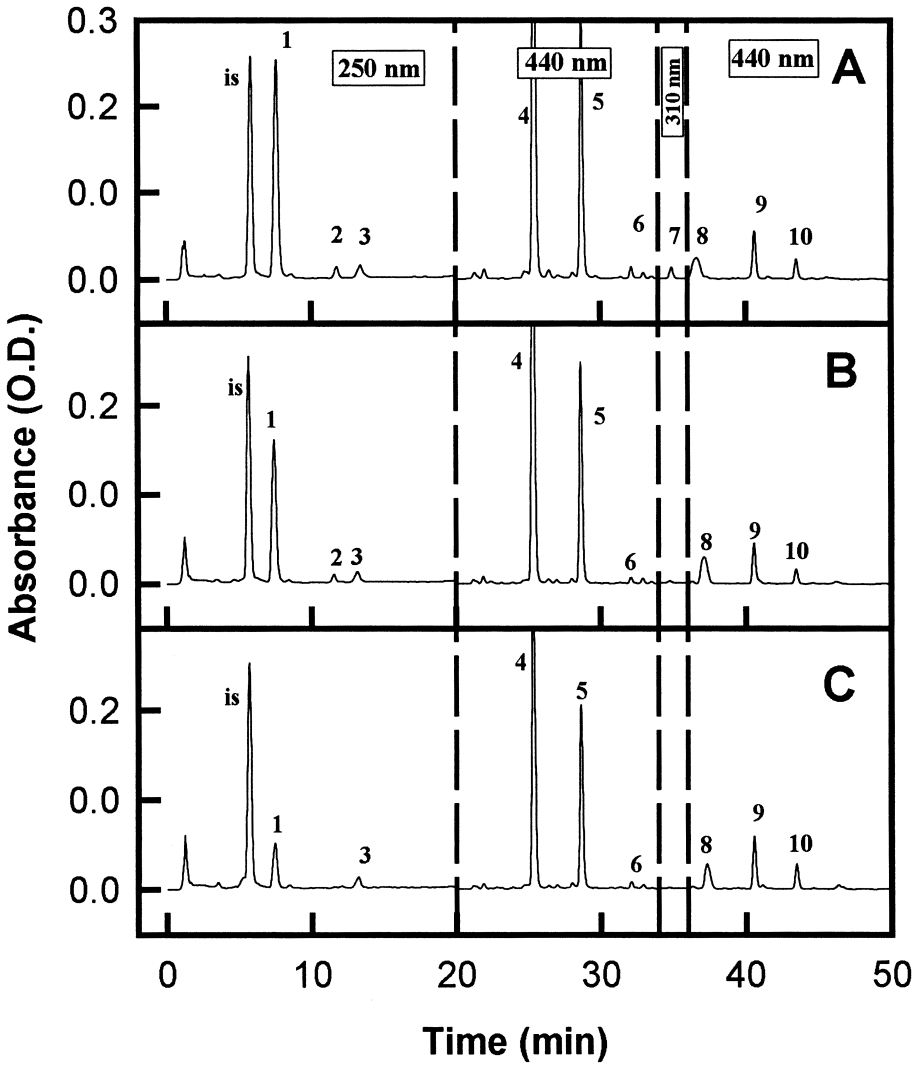

Fig. 2. Chromatograms of methanol:water (50%, v/v) saffron extracts from Mancha (A), Rio (B) and Sierra (C) types, simultaneously recorded at 250, 310 and 440 nm, including 4-nitroaniline as internal standard. The mobile and stationary phases were a lineal gradient of methanol–water from 20 to 70% in 50 min, and a Millipore-Novapack RP-C18, respectively. The flow-rate was 1 ml/min, temperature 30°C and sample size 50 μl. The following compounds are present: picrocrocin (1), HTCC (2), 3-gentiobiosil-kaempferol (3), α-crocin (4), crocin 2 (5), crocin 3 (6), safranal (7), crocin 4 (8), crocin 5 (9), crocin 6 (10) and internal standard (I.S.).

even lower than the Rio type sample. These results agreed with the Spanish saffron classification by quality and price. This classification is based on stigma length, colour, odour and the presence of floral rest [6], but the concentration of each saffron metabolite has not been usually considered, probably due to the lack of an appropriate quantitative method of analysis. In this way, the present work can be a contribution to improve the analysis of saffron. The HPLC-UV procedures show adequate analytical performance and can possibly become a standard method for saffron analysis.

Table 1
Chromatographic parameters of saffron metabolites and adulterants contained in 1 mg/ml saffron extract (Mancha type) with 0.02 mg/ml of each adulterant (50 μl sample size), analysed by HPLC under the conditions described in Fig. 1

	Compound	Wavelength (nm)	A_s	k'	R_s
Adulterants and I.S.	Tartracin	440	0.7	0.04	
					2.0
	Ponceau S	440	0.4	1.0	
					6.4
	4-Nitroaniline	250, 310, 440	1.1	4.6	
					1.2
	Methyl orange	440	1.2	5.2	
					2.2
Saffron metabolites	Picrocrocin	250	1.3	6.4	
					7.8
	HTCC	250	1.0	10.4	
					3.1
	Kaempferol	250	0.9	2.3	
					18.5
	α-Crocin	440	1.0	24.0	
					6.8
	Crocin 2	440	1.5	27.2	
					7.9
	Crocin 3	440	1.3	30.6	
					6.1
	Safranal	310	1.0	33.2	
					3.4
	Crocin 4	440	0.9	36.0	
					3.5
	Crocin 5	440	1.0	38.9	
					6.1
	Crocin 6	440	1.0	41.7	

Table 2
Saffron metabolite concentrations and the ratios between them in the three Spanish saffron quality samples (Mancha, Río and Sierra), and a Mancha commercial sample, analysed by HPLC under the conditions described in Fig. 1

Wavelength (nm)	Compound	Mancha ($A_{Comp}/A_{I.S.}$)	Rio ($A_{Comp}/A_{I.S.}$)	Sierra ($A_{Comp}/A_{I.S.}$)	Commercial sample ($A_{Comp}/A_{I.S.}$)
250	Picrocrocin	1.23	0.75	0.22	0.51
	HTCC	0.05	0.03	n.d.	0.04
	Kaempferol	0.08	0.06	0.05	0.04
310	Safranal	0.06	n.d.	n.d.	0.03
440	α-Crocin	3.85	2.30	2.10	3.43
	Crocin 2	1.81	1.01	1.15	1.48
	Crocin 3	0.07	0.02	0.04	0.08
	Crocin 4	0.40	0.25	0.26	0.32
	Crocin 5	0.32	0.21	0.32	0.39
	Crocin 6	0.14	0.07	0.16	0.15

n.d., not detected.

4. Nomenclature

HTCC	2,6,6-trimethyl-4-hydroxy-1-carboxaldehyde-1-cyclohexene
Kaempherol	3-gentiobiosil-kaempherol
$A_{Comp}/A_{I.S.}$	compound area/internal standard area
$A_{Picro}/A_{I.S.250}$	picrocrocin area at 250 nm/internal standard area at 250 nm
$A_{\alpha\text{-}Cro}/A_{I.S.250}$	α-crocin area at 440 nm/internal standard area at 440 nm

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