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Preparative high-performance liquid chromatographic purification of saffron secondary metabolites

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ABSTRACT

A preparative HPLC procedure to isolate picrocrocin, the compound responsible for the taste of saffron and precursor of the aromatic safranal, and the mixture of yellow pigments from a saffron hydroalcoholic extract has been developed. A reversed-phase C_{18} column was employed as the stationary phase. The best separation was obtained with 45% methanol, plus a 90% acetonitrile pulse 3 min after starting the run, as mobile phase. To obtain the highest yield from the system, sample size was increased up to 2 ml of 200 mg ml^{-1} saffron extract; under such conditions a good resolution was obtained and picrocrocin and saffron pigments were separated with a high purification yield and purity.

INTRODUCTION

Saffron is a very high-value spice obtained from dried *Crocus sativus* stigmas. It is mainly employed to provide colour and flavour to foods [1,2]. The most important secondary metabolites in saffron are picrocrocin and crocetin glycosyl esters. Picrocrocin is a colourless bitter glycoside, responsible for the bitter taste of saffron and precursor of safranal, the saffron aromatic. This latter compound can be obtained from picrocrocin by chemical or enzymatic hydrolysis [1,3–6]. In fact, picrocrocin has potential value as a food additive, to provide aroma and flavour. The yellow–red pigments of saffron are a mixture of glycosides derived from the polyene dicarboxylic acid crocetin, in which glucose and gentobiose occur as carbohydrate residues. Of this group of substances the digentobiosyl ester of crocetin, namely crocin, is the most abundant; other crocetin derivatives are designated crocin ester 1 [crocetin (β-D-gentobiosyl)-(β-D-glucosyl) ester] and crocetin ester 2 [crocetin-mono-(β-D-gentobiosyl) ester] [1,3,7,8]. The major components of this mixture of pigments are freely soluble in water, which is an important advantage of the mixture for its use as a colour additive in the food and pharmaceutical industries.

Analytical separation of saffron secondary metabolites by TLC [4,9,10] and HPLC [2,8, 11,12] has been described. All these studies were carried out for analytical purposes, but none of them developed a preparative system for the purification of saffron secondary metabolites. In a previous study in our laboratory, isolation of picrocrocin and pigments by preparative TLC was achieved [13]. Purification results obtained gave a good chromatographic purity and high isolation yield for picrocrocin. However, the operation time was long and the pigments were strongly retained on the support, resulting in a low purification yield. The aim of this work was

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to develop a simple and reproducible method to isolate picrocrocin and the mixture of yellow pigments from saffron by preparative HPLC.

EXPERIMENTAL

Materials

Dried saffron type “Mancha Superior” was purchased from a local supplier and stored in the dark at 4°C. Acetonitrile and methanol of HPLC grade were from Romil. Water was double distilled and filtered through a Millipore system (Milli-Q). All others reagents were analytical grade and were used without further purification.

Sample preparation

Crude extract was obtained from saffron as previously described [13]. Saffron was ground, resuspended in 50% ethanol and stirred; the vegetal tissue residue was discarded after centrifugation. The hydroalcoholic extract obtained was vacuum concentrated, and the aqueous solution obtained was lyophilized and stored at −20°C until use. Samples of extract (from 10 to 200 mg ml^{-1}) were prepared by dissolving the lyophilized extract in water.

Method development

Preparative HPLC analyses were performed on a Shimadzu HPLC system equipped with two LC-8A pumps, injectors for analytical and preparative runs, a UV–Vis spectrophotometric detector (SPD-6AV) with analytical and preparative flow cells, an FCV-100B fraction collector and a C-R4A Chromatopac integrator. Columns used were 25 cm × 0.46 cm I.D. and 25 cm × 2.12 cm I.D. (Supelco) for the analytical-scale (referred to below as the small-scale column) and the preparative-scale assays, respectively, both packed with Supelcosil PL C-18 (12 μm particle size). The standard mobile phase used for preparative separation of picrocrocin and pigments was isocratic 45% methanol, plus a 6-s pulse of 90% acetonitrile 3 min after starting the run. The sample solution was manually injected with a syringe. Flow-rates were 2 and 42 ml min^{-1} and sample sizes 20 μl and 2 ml for small-scale and preparative assays, respectively. Picrocrocin was detected at 250 nm and pigments at 440 nm for small-scale assays. As crocin elution could also be detected at 250 nm, for preparative runs all compounds were monitored at 250 nm. All separations were performed at room temperature.

The identification of saffron secondary metabolites was made by HPLC using commercial crocin and previously purified picrocrocin [13] as standards. Picrocrocin and crocin were quantified using their molar absorptivities in water: $\epsilon_{250} = 10\,100$ M^{-1} cm^{-1} [2] and $\epsilon_{440} = 133\,750$ M^{-1} cm^{-1} [14], respectively.

Purity analysis

The purity of purified compounds was determined by analytical HPLC using a Shimadzu LC-6A chromatograph, equipped with a 150 mm × 4 mm I.D. Lichrosolv RP C_{18} (5 μm particle size) column from Merck, an automatic injector (Shimadzu SIL 9A) and a photodiode-array UV–Vis detector (Shimadzu SPD-M6A). The mobile phase employed was a linear gradient of acetonitrile (20% to 55% within 10 min) in water, sample size was 20 μl and flow-rate 1 ml min^{-1}. Purification yield of picrocrocin was determined by the ratio of the amount of picrocrocin injected into the preparative column and that recovered after purification. For the pigments, only identification and quantitation of crocin was possible, so the purification yield of pigments was referred to as crocin yield, after checking that in the purified pigments there were no qualitative changes, that is the peak-area ratios of the different compounds of the pigment mixture were the same before and after the purification process. The chromatographic purity was determined as the percentage of peak area corresponding to each compound with reference to the total area integrated for all peaks in the chromatogram.

RESULTS AND DISCUSSION

A C_{18} stationary phase, previously used for the separation of saffron secondary metabolites by different authors [2,8,11,12], was selected. The most suitable mobile phase was determined

after an analysis employing the small-scale section of the preparative HPLC equipment. Several binary and ternary isocratic solvent systems consisting of combinations of methanol, acetonitrile and water were assayed as eluents. Of these, 50% (v/v) methanol was selected as optimum mobile phase for picrocrocin and pigments separations, obtaining an analysis time shorter than 8 min, with capacity factor (k') values of 1.4, 2.8 and 7.2 for picrocrocin, crocin and crocetin ester 1, respectively. The selectivity coefficients (α) and resolution (R_s) values between picrocrocin and crocin were 2.28 and 3.46, and between crocin and crocetin ester 1, 1.92 and 3.32, respectively.

These separation conditions were applied to the preparative column, keeping constant the linear eluent velocity [15], which meant increasing the flow-rate from 2 to 42 ml min^{-1}. However, it was not possible to elute the components with the same resolution, because the retention time was longer for all the components. Thus, slight changes in the organic solvent concentration of the mobile phase had to be made. With the methanol concentration adjusted to 55%, retention times were 2.1, 3.3 and 5.0 min for picrocrocin, crocin and crocetin ester 1, respectively; the remaining, more hydrophobic, pigments were retained in the column, and their elution with 55% methanol required the operation time to be extended by up to 25 min. Under these conditions it was possible to obtain pure picrocrocin and crocin, which can be used as standards.

A modification of the elution system consisting in a reduction in the methanol concentration from 55% to 45%, which increased the retention time of pigments, and giving a 90% acetonitrile pulse for 6 s 3 min after starting run, to facilitate the elution of all the remaining pigments, was assayed. Thus, picrocrocin was obtained at a retention time of 2.8 min, and all the pigments together, in the same fraction, at 5.2 min. The run was finished after 7 min and initial conditions achieved. Separation between these fractions was about 2 min, which allowed an increase in the amount of injected sample for every run. Fig. 1 shows preparative HPLC chromatograms for this latter system; picrocrocin was peak number 6, while pigments were peaks numbers 9 and 10.

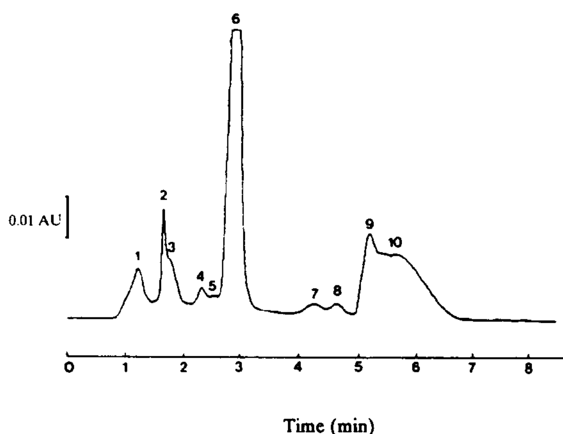

Fig. 1. Chromatograms of preparative separation of 10 mg ml^{-1} saffron extract with 45% methanol, plus a 90% acetonitrile pulse 3 min after starting the run, as the mobile phase (see text for peak identification).

To obtain the highest yield of picrocrocin and crocin with the preparative HPLC method developed, the separation was assayed with 2 ml of 10, 50, 100, 150 and 200 mg ml^{-1} saffron extract. According to the detector response, monitored at 250 nm, fractions of picrocrocin and pigments were collected from 2.6 to 3.2 min and from 4.9 to 6.5 min, respectively. These collected fractions were analysed, after concentration, by an analytical HPLC system equipped with a photodiode array UV–Vis detector to determine chromatographic purity. Chromatograms obtained are shown in Fig. 2. Peak numbers 6, 7 and 9 are picrocrocin, crocin and crocetin ester 1, respectively; purified picrocrocin fraction (a) showed only one peak and no other contaminant compounds were detected in significant amounts, while the pigments fraction showed peaks corresponding to crocin and crocetin ester 1 and other yellow pigments derived from crocetin.

Purification yields of 80% and 99% were achieved for picrocrocin and pigments, respectively, and no significant changes in these values were obtained for the different sample sizes assayed. Chromatographic purity for picrocrocin was always over 95%. An increase in picrocrocin purification yield was difficult due to the appearance of a minor yellow pigment, more hydrophilic than crocin, which was eluted slightly

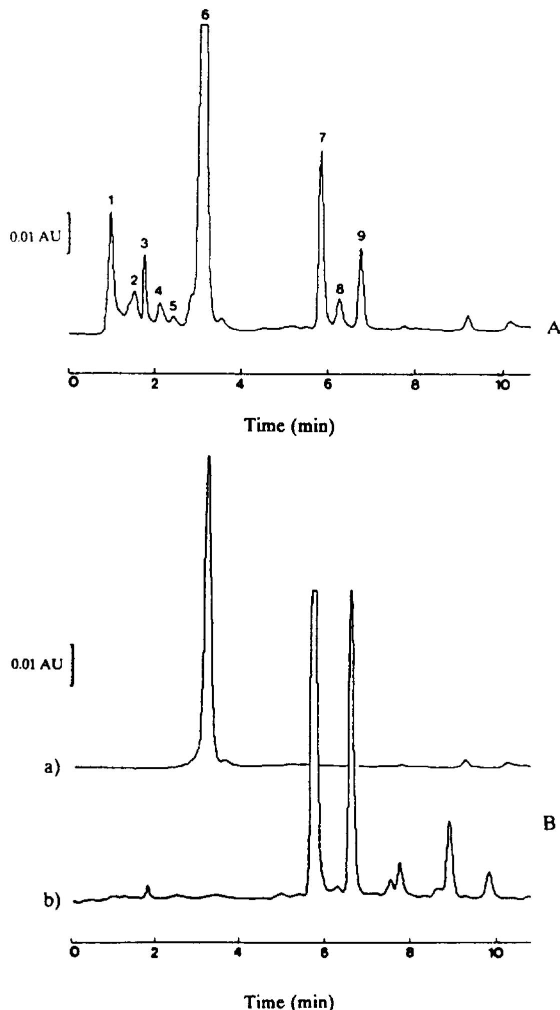

Fig. 2. Analytical HPLC chromatograms before and after the purification process. (A) Saffron extract at 250 nm and (B) picrocrocin fraction at 250 nm (a) and pigments mixture fraction at 440 nm (b) (see text for peak identification).

before picrocrocin. Thus, to maintain a chromatographic purity of 95% the purification yield had to be reduced to 80%. The purified compounds were lyophilized before storage and no degradation was observed after 6 months' storage at −20°C.

Finally, the system employed allows the injection and elution process to be automated. Thus 2 ml of 200 mg ml^{-1} saffron extract could be injected into the column every 8 min, *i.e.* 7.5 runs every hour of continuous operation. This estimation means that 0.51 g h^{-1} picrocrocin could be obtained.

In conclusion, a simple, efficient and reproducible method for the purification picrocrocin and saffron pigments by preparative HPLC has been developed. In addition, the results reported in the present work are a contribution to the possible industrial use of picrocrocin and saffron pigments separately.

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