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Analytical Methods

Solid-phase extraction for picrocrocin determination in the quality control of saffron spice (*Crocus sativus* L.)

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ABSTRACT

The application of solid phase extraction (SPE) in the determination of picrocrocin by UV–vis spectrophotometry has been studied in order to develop a rapid and low cost method that can be used in the industry for routine quality control of saffron spice. Parameters influencing the SPE procedure, such as concentration of the initial extract, sample size and eluents, were studied and optimized. Twenty different saffron spice samples from Greece, Iran, Italy and Spain were used in the intra-laboratory validation of the SPE method. The results indicated the selectivity, trueness, linearity, precision (repeatability: RSD < 6%, intermediate precision: RSD < 10%), good recovery (about 90%) and sensitivity (LOD = 0.30 mg L^{-1}; LOQ = 0.63 mg L^{-1}). The method also proved valid for overcoming the limitations of $E^{1\%}_{1cm}$ 257 nm due to crocetin esters in the determination of picrocrocin.

1. Introduction

Saffron spice, made up of the dried stigmas of *Crocus sativus* L., is chiefly used in food due to its colouring, flavouring and aromatic properties. Saffron quality in the international trade has been mainly determined by specifications recommended by the ISO 3632 standard, whose latest revision has given rise to the Technical Specification ISO/TS 3632 (2003). This classifies saffron into three categories with regard to a large number of physical and chemical parameters that define saffron quality: microscopic characteristics, presence of flower waste, moisture and volatile matter content, ash content, $E^{1\%}_{1cm}$ 440 nm (colouring strength), $E^{1\%}_{1cm}$ 330 nm, $E^{1\%}_{1cm}$ 257 nm, etc. These last three parameters are historically related to the content of crocetin esters, safranal and picrocrocin, respectively. The crocetin esters are a group of water-soluble carotenoids that derive from crocetin ($C_{20}H_{24}O_4$, 8,8′-diapo-Ψ,Ψ′-carotenedioic acid), where glucose, gentiobiose, neapolitanose or triglucose are the sugar moieties and where trans- or cis-configuration is found. This group of compounds is responsible for colour and colouring properties of saffron spice and represents between 0.5% and 32.4% on a dry basis of saffron spice (Alonso, Salinas, Garijo, & Sánchez, 2001). Safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) is the major compound in the volatile fraction of saffron representing around 70% (Carmona, Zalacain, Salinas, & Alonso, 2007; Rödel & Petrizka, 1991; Tarantilis & Polissiou, 1997; Zarghami & Heinz, 1971). Picrocrocin (4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde), with reported contents in saffron spice from 0.8% to 26.6% on a dry basis (Alonso et al., 2001; Iborra, Castellar, Canovas, & Manjón, 1992; Sánchez et al., 2008b), is considered responsible for saffron's bitter taste.

The systematic use of the specifications recommended by the ISO 3632 has managed to classify saffron in world trade by its colouring strength, provided that the remaining requirements are fulfilled. Consequently, this classification has led to the existence of a spectrophotometer in almost all saffron companies. The colouring strength is representative of the crocetin ester content. However, the determination of picrocrocin through the parameter $E^{1\%}_{1cm}$ 257 nm shows a problem of selectivity since other compounds of saffron extract, primarily crocetin esters, also have absorbance at this wavelength due to the glycoside bonds, causing interferences in measurement (Carmona, Zalacain, Sánchez, Novella, & Alonso, 2006; Orfanou & Tsimidou, 1996; Sánchez et al., 2008b; Tarantilis, Polissiou, & Manfait, 1994).

Up to now, other techniques such as thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) have been used to quantify picrocrocin, with this last technique considered as being the most effective (Alonso et al., 2001; Lozano, Castellar, Simancas, & Iborra, 1999; Sujata, Ravishankar, & Venkataraman, 1992; Tarantilis, Tsoupras, & Polissiou, 1995). Near-infrared (NIR) spectroscopy has also been applied to the determination of saffron chemical composition (Zalacain et al., 2005b). However, all of these methods can hardly be used for routine industrial work

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to monitor raw materials, processes or final products since they are time consuming and, in the case of HPLC or NIR spectroscopy, require equipment that is seldom found in small or medium-size companies that process and package saffron spice. Thus, there is a real interest in the development of rapid methods for routine quality control of saffron using UV–vis spectral information (Sánchez et al., 2008b; Zalacain et al., 2005a). Definitively, the main requirements that such methods should fulfil are the following: (a) to overcome the limitations of $E^{1\%}_{1cm}$ 257 nm to determine picrocrocin from saffron by avoiding interferences from other saffron compounds, mainly crocetin esters; (b) low cost, which first implies no investment in additional equipment and secondly, the use of small quantities of the sample; and (c) rapidity and straight-forwardness.

Solid phase extraction (SPE) is one of the most common and least expensive purification techniques and is considered as a convenient approach for sample preparation in food analysis. In the last few years much research has gone into SPE application to the analysis of major and minor components of foods (Grigoriadou, Androulaki, Psomiadou, & Tsimidou, 2007; Puoci et al., 2008). In saffron analysis, SPE is applied to the detection of adulterations by artificial colorants (ISO, 2003; Zalacain et al., 2005a). However, it has only been marginally applied to saffron component purification (Carmona et al., 2007; Escribano, Alonso, Coca-Prados, & Fernández, 1996; Sujata et al., 1992).

The purpose of this work was to study the application of SPE in order to avoid crocetin ester interferences in the determination of picrocrocin by UV–vis spectrophotometry during the routine quality control of saffron spice. Parameters influencing the SPE procedure such as concentration of the initial extract, sample size and eluents were studied and optimized. The SPE method was validated and applied to different saffron spice samples from Greece, Iran, Italy and Spain.

2. Materials and methods

2.1. Samples and chemicals

Twenty one saffron spice samples in filaments were used. These were obtained directly from the producers and packers with a guarantee of their origin and freedom from fraud (Table 1). They came from Greece, Iran, Italy and Spain, and were harvested during the years 2004, 2005 or 2006. All Spanish samples from the 2006 harvest were of the Protected Designation of Origin "Azafrán de La Mancha". Picrocrocin purified according to the method described below was used for calibration curves. C_{18} adsorbent (125×10^{-8} cm pore size, 55–105 μm particle size) for picrocrocin isolation was from Waters (Milford, MA). HPLC-grade acetonitrile and cyclohexane were used from Scharlau (Barcelona, Spain). Ultra high purity water was produced using a Milli-Q System from Millipore (Bedford, MA) and PTFE filters (11 mm, 0.45 μm) were also purchased from Millipore. C_{18} SPE cartridges (Sep-pak plus™, 125×10^{-8} cm pore size, 55–105 μm particle size, 360 mg sorbent weight) were supplied by Waters, (Milford, MA).

2.2. Picrocrocin isolation

Picrocrocin was extracted from saffron and isolated by column chromatography using a C_{18} adsorbent. For extraction, 30 mL of cyclohexane were added to 5 g of powdered saffron and the suspension was left in the dark at room temperature for 24 h with sporadic agitation. Then the organic solvent was discarded and the solid residue was dried under reduced pressure. Sixty milliliters of nitrogen-saturated water were added to the thus treated saffron and the resulting suspension was stirred for 1 h in the dark at room temperature. Then the extract was centrifuged at 4000 rpm for 10 min and the supernatant was collected and loaded onto the previously conditioned C_{18} column (8 cm high × 2.7 cm i.d.). Picrocrocin was eluted with 90 mL of 10% acetonitrile/water (v/v) after the elution of flavonoids with 20 mL of 2% acetonitrile/water (v/v). Finally, the solvent was eliminated by evaporation to dryness under reduced pressure and the purified picrocrocin was kept at −20 °C until its utilization. The chromatographic purity of the obtained picrocrocin was 96%, calculated as the percent of the total peak area at 250 nm.

2.3. Saffron extract preparation

Aqueous extracts of 2.5 g L^{-1} and 0.5 g L^{-1} concentration were prepared with ultra high purity water by stirring the suspension in the dark at room temperature for 1 h, as specified by the ISO (2003). The former were used in the study of the retention and elu-

Table 1
Origin, harvest and quality characteristics of the saffron samples according to ISO/TS 3632 (2003).

Sample	Origin	Harvest	Moisture and volatile matter content (%)	$E^{1\%}_{1cm}$ 440 nm (mean ± SD)[a]	$E^{1\%}_{1cm}$ 330 nm (mean ± SD)	$E^{1\%}_{1cm}$ 257 nm (mean ± SD)	Category
1	Greece	2005	8.3	234.4 e ± 2.0	41.3 i ± 0.6	83.4 e ± 1.1	I
2	Greece	2005	8.5	273.0 i,j ± 1.6	36.4 c,d,e,f ± 0.8	99.2 i,j ± 1.4	I
3	Greece	2005	8.9	255.7 g ± 1.1	39.7 g,h,i ± 0.5	93.7 f,g ± 1.4	I
4	Greece	2005	8.2	259.7 g,h ± 6.4	35.5 b,c,d ± 0.9	94.9 f,g,h ± 2.3	I
5	Greece	2005	10.4	162.6 a ± 4.6	41.3 i ± 1.1	71.4 a,b ± 2.0	II
6	Iran	2006	8.7	233.7 e ± 2.3	40.4 h,i ± 0.4	86.3 e ± 1.0	I
7	Iran	2006	9.6	267.0 h,i ± 3.1	37.8 e,f,g,h ± 2.4	97.7 h,i ± 3.1	I
8	Iran	2006	8.8	170.5 b ± 4.2	41.6 i ± 1.0	74.1 b,c ± 1.8	II
9	Iran	2006	6.9	232.9 d,e ± 1.7	37.7 d,e,f,g ± 4.5	93.5 f,g ± 4.5	I
10	Iran	2005	7.2	199.7 c ± 4.9	38.5 f,g,h ± 0.9	79.4 d ± 1.9	I
11	Italy	2006	8.4	266.1 h,i ± 8.2	33.8 b ± 1.8	97.2 g,h,i ± 3.5	I
12	Italy	2006	9.0	284.3 k ± 7.0	35.0 b,c ± 0.9	101.9 j ± 2.5	I
13	Italy	2006	8.8	199.1 c ± 2.1	36.1 b,c,d,e ± 0.8	79.4 d ± 0.7	I
14	Italy	2006	10.1	225.6 d ± 7.7	41.8 i ± 1.8	92.2 f ± 3.5	I
15	Spain	2006	6.1	258.5 g ± 7.2	29.9 a ± 1.0	93.4 f,g ± 2.5	I
16	Spain	2006	6.4	276.1 j ± 6.8	30.9 a ± 0.8	95.4 f,g,h ± 2.4	I
17	Spain	2006	6.4	230.7 d,e ± 1.2	47.0 j ± 0.1	76.3 c,d ± 0.2	I
18	Spain	2006	6.0	245.2 f ± 6.0	40.0 g,h,i ± 1.0	93.3 f ± 2.3	I
19	Spain	2004	7.7	162.4 a ± 6.9	40.4 h,i ± 1.6	70.0 a ± 3.1	II
20	Spain	2004	8.0	198.9 c ± 5.5	38.8 g,h,i ± 1.5	84.2 e ± 1.1	I
21	Spain	2006	6.1	259.3 ± 3.1	29.4 ± 1.4	99.2 ± 2.3	I

[a] For samples 1–20, the same letter in a column indicates non significant differences according to Duncan's test at the 0.05% level. Sample 21 was used for the evaluation of SPE cartridge behaviour.

tion of saffron components in the SPE cartridges, while the latter were used for the determination of the main characteristics of saffron and in the optimized SPE procedure. The extracts were centrifuged at 4000 rpm for 5 min before being loaded into the SPE cartridges.

2.4. Solid-phase extraction procedure

The C_{18} SPE cartridges were conditioned with 2 mL of acetonitrile, followed by 5 mL of water, 2 mL of acetonitrile and 5 mL of water before the application of the saffron extract. Four milliliters of a saffron aqueous extract (2.5 g L^{-1}) were loaded into the SPE cartridge. Then, to study the retention and elution characteristics of the cartridge, this was washed successively with water, 5% and 15% acetonitrile/water (v/v). For each solvent, eluted fractions of 3 mL were collected and their absorbances at 250 and 440 nm were measured until they were nearly zero. SPE procedure was optimized with respect to sample size: 7 mL, 4 mL, 2 mL and 1 mL of a 0.5 g L^{-1} extract; and elution solvent: 5%, 10%, 12% and 15% of acetonitrile/water (v/v).

The optimized SPE procedure for picrocrocin purification was as follows: 1 mL of the 0.5 g L^{-1} saffron extract was added to the SPE cartridge, it was washed with 10 mL water, and then picrocrocin was eluted with acetonitrile/water 12% (v/v) up to collecting 10 mL into a volumetric flask being measured. Finally, to check the composition of all fractions, crocetin esters were eluted with 10 mL of acetonitrile.

2.5. Spectrophotometric analysis

Spectral characteristics of aqueous saffron extracts and eluted fractions were monitored by scanning from 190 to 700 nm using a Perkin–Elmer Lambda 25 spectrophotometer (Norwalk, CT, USA) with UV WinLab 2.85.04 software (Perkin–Elmer). Colouring strength ($E^{1\%}_{1cm}$ 440 nm), $E^{1\%}_{1cm}$ 257 nm and $E^{1\%}_{1cm}$ 330 nm were determined according to ISO (2003). $E^{1\%}_{1cm}$ 250 nm was assessed from the absorbance at 250 nm of the fraction containing picrocrocin. ΔEpic was calculated from spectral data of saffron extracts as defined by Corradi and Micheli (1979):

$$\Delta E\text{pic} = E^{1\%}_{257} - E^{1\%}_{297} \quad (1)$$

All analyses were done in duplicate and two measurements were also taken for each replicate.

2.6. RP-HPLC-DAD analysis

Twenty microliters of each sample (aqueous saffron extracts and eluted fractions) were injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) operating with a 150 mm × 4.6 mm i.d., 5 μm Phenomenex (Le Pecq Cedex, France) Luna C_{18} column, thermostated at 30 °C. Eluents were water (A) and acetonitrile (B) with the following gradient: 20% B, 0–5 min; 20–80% B, 5–15 min; 80% B, 15–20 min; 20% B, 20–30 min. The flow rate was 0.8 mL min^{-1}. The DAD detector (Hewlett Packard, Waldbronn, Germany) was set at 250 and 440 nm for picrocrocin and crocetin ester detection, respectively. All analyses were done in duplicate and two measurements were also taken for each replicate.

2.7. Quantification of picrocrocin and crocetin esters

From the purified picrocrocin, solutions of seven different concentrations: 0.51, 2.56, 5.12, 10.24, 25.60, 51.20 and 256.00 mg L^{-1} were prepared in water while solutions of six concentrations: 0.60, 3.00, 6.00, 12.00, 30.00 and 60.00 mg L^{-1} were prepared in acetonitrile/water 12% (v/v). Quantification of picrocrocin was accomplished with the use of the calibration curves calculated by linear regression analysis. Quantification of crocetin esters was done as previously reported by Sánchez, Carmona, Ordoudi, Tsimidou, and Alonso (2008a).

2.8. Validation of the SPE method

Intra-laboratory method validation was carried out according to Eurachem Guidelines (Eurachem, 1998). The selectivity of the method to determine picrocrocin was studied by measuring the SPE fraction that contained picrocrocin with HPLC and checking that no additional peaks appeared in the chromatograms. Accuracy was studied as two components: trueness and precision. Trueness was assessed as the closeness of agreement between the average content of picrocrocin for the same samples obtained after SPE, and the reference value of picrocrocin content determined by direct injection of saffron extracts in the HPLC chromatograph. With regard to precision, two parameters were determined: repeatability and intermediate precision and they were stated in terms of relative standard deviation (RSD). Repeatability was checked for the sample numbered as 15 (Table 1) analysed five times by the same analyst within the same day. The repeatability limit, L_r, at the 95% confidence level was calculated as:

$$L_r = 1.96 \times \sqrt{2} \times \sigma_r \quad (2)$$

where σ_r is the standard deviation measured under repeatability conditions.

Intermediate precision was determined for the same saffron sample that was used to calculate repeatability. This sample was analysed by different analysts on three separate days. The reproducibility limit, L_R, at the 95% confidence level was calculated as:

$$L_R = 1.96 \times \sqrt{2} \times \sigma_R \quad (3)$$

where σ_R is the standard deviation measured under reproducibility conditions.

Besides sample 15, samples numbered as 1, 2, 9, 13 and 19 (Table 1) were used in the recovery study of picrocrocin in order to broaden the concentration range. Limit of detection (LOD) was calculated as the picrocrocin concentration corresponding to the mean value of 10 independent sample blanks measured once each plus three times their standard deviation. As it was not possible to have a sample of saffron without picrocrocin, water was used as a blank sample. Limit of quantification (LOQ) was calculated as the picrocrocin concentration corresponding to the mean value of 10 independent sample blanks measured once each, plus 10 times their standard deviation.

Linearity for both the UV–vis method and the HPLC one was determined by plotting signal response versus picrocrocin concentration in water and acetonitrile/water 12% v/v in the range of 256.00–0.51 mg L^{-1} using at least six levels of calibration.

Data were subjected to a comparison of means with a Student t test ($\alpha = 0.01$) and analysis of variance (ANOVA) using the SPSS 15.0 statistical program for Windows (SPSS Inc., IL, USA).

3. Results and discussion

3.1. Evaluation of SPE cartridge behaviour

A 2.5 g L^{-1} extract was prepared with the saffron sample numbered as 21 (Table 1) from the Designation of Origin "Azafrán de La Mancha". In Fig. 1 the retention and elution characteristics of the SPE cartridges loading 4 mL of the saffron extract are shown. They were monitored at 250 and 440 nm because these are wavelengths close to picrocrocin and crocetin ester absorbance maxima, respec-

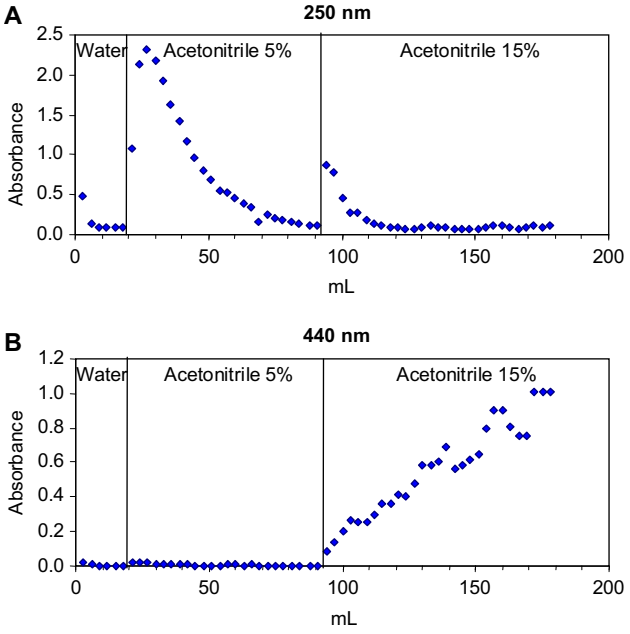

Fig. 1. Retention and elution characteristics of the SPE cartridges. Absorbance at 250 nm (A) and 440 nm (B) measured in 3 mL fractions after loading 4 mL of a 2.5 g L^{-1} saffron extract.

tively. It was observed that very few substances were eluted with water. The compounds that absorb at 250 nm (Fig. 1A) started to elute with acetonitrile/water 5% (v/v) and after 73 mL of this solvent, the absorbance of the eluted fractions was nearly zero, suggesting that most of picrocrocin had been eluted. An increase of absorbance at 250 nm followed by a steady decrease until reaching a plateau was observed when acetonitrile/water 15% (v/v) was added, showing the picrocrocin that remained in the cartridge. Nearly 200 mL of acetonitrile/water 15% (v/v) were necessary for complete elution of crocetin esters (Fig. 1B). These results suggested a reduction of the extract concentration and a light increase in the proportion of acetonitrile for the elution of picrocrocin in order to use lower elution volumes and increase picrocrocin recovery, which are crucial factors for a rapid and low cost method.

3.2. Optimization of the SPE procedure

The concentration of the extract to be loaded into the SPE cartridge was established as 0.5 g L^{-1} so that the same extract used in the determination of the colouring strength could be used in the proposed method, with the subsequent saving of time and work.

Regarding the sample size, the maximum one that the SPE cartridge was able to hold was 7 mL, but there was not a good separation of picrocrocin and crocetin esters. With 4 mL the separation improved, although too much eluent was still necessary to elute all the picrocrocin. Two and 1 mL showed the best results, and we decided to use the latter in order to shorten the time of analysis. A volume of 10 mL of water was sufficient for the clean-up step, while 10 mL of acetonitrile/water 12% (v/v) were necessary to manage the elution of picrocrocin without that of crocetin esters. Lower acetonitrile concentrations (5%) resulted in too many losses of picrocrocin in the SPE cartridge, as previously mentioned, while higher ones (15%) resulted in a contamination of the picrocrocin fraction with crocetin esters. In Fig. 2 the UV–vis spectra of the two eluted fractions containing the picrocrocin and crocetin esters respectively are shown and compared to that of the saffron extract from which they came. As could be observed when comparing the

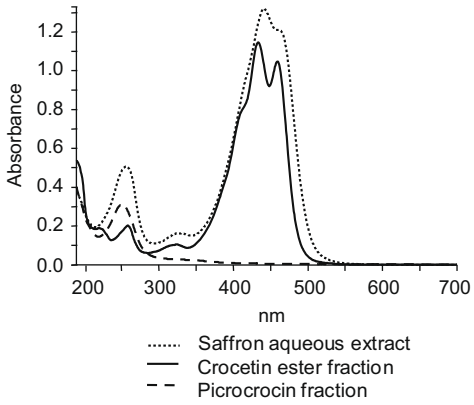

Fig. 2. UV–vis spectra of a saffron extract and its corresponding crocetin ester and picrocrocin fractions after SPE.

spectrum of the picrocrocin fraction to that of crocetin esters, both crocetin esters and picrocrocin absorb at 257 nm. Consequently, absorbance of saffron extracts at this wavelength is influenced not only by picrocrocin content but also by crocetin ester composition.

Another point to be taken into account is that $E^{1\%}_{1cm}$ 440 nm and $E^{1\%}_{1cm}$ 257 nm are measured, according to ISO (2003), after a dilution 1/10 of a 0.5 g L^{-1} extract. Therefore, the 10 mL volume of the eluted picrocrocin fraction also supposed a dilution of 1/10 with respect to the loaded extract (1 mL). This allows for the use of the same factor which multiplies the corresponding absorbance to express the results of colouring strength ($E^{1\%}_{1cm}$ 440 nm), $E^{1\%}_{1cm}$ 257 nm and also the results obtained with the SPE method developed. Our proposal is to express the content of picrocrocin as $E^{1\%}_{1cm}$ 250 nm of the fraction in which it is contained, firstly because the companies are familiarized with the $E^{1\%}_{1cm}$, and secondly, because the conversion to percentage on a dry basis of saffron, once known the molar absorption coefficient, ε, of picrocrocin is immediate:

$$\%\ \text{of picrocrocin on dry basis} = \frac{\left(E^{1\%}_{1cm}250\,\text{nm}\right) \times \text{Mw} \times 10}{\varepsilon} \times F \qquad (4)$$

where Mw is the molecular weight of picrocrocin, and F is a factor included in order to correct the results according to the recovery of picrocrocin in the SPE procedure.

3.3. Validation of the SPE method

Table 1 displays the main saffron quality characteristics according to ISO (2003) of the 20 samples used for the intra-laboratory validation. A total of 17 samples fulfilled the specifications for the best category: category I, regarding moisture and volatile matter content, as well as the main characteristics using UV–vis spectrophotometry, whereas the rest of the samples belonged to category II. The moisture and volatile matter content of the samples studied ranged from 6.0% to 10.4%, the colouring strength ranged from 162.4 to 284.3, $E^{1\%}_{1cm}$ 330 nm ranged from 29.4 to 47.0 and $E^{1\%}_{1cm}$ 257 nm from 70.0 to 101.9.

The sample composition in crocetin esters and picrocrocin is shown in Table 2. As previously reported by Sánchez, Carmona, Ordoudi, Tsimidou, and Alonso (2008a), the samples with the highest or the lowest crocetin ester contents were the samples having the highest or the lowest $E^{1\%}_{1cm}$ 440 nm as well, i.e. samples 12 and 19, respectively (Table 1). However, our results revealed that there was no coincidence between the sample with the highest or lowest content of picrocrocin (sample 4 or sample 17, respectively)

Table 2
Composition in crocetin glycosides and picrocrocin; Δ*E*pic; and $E^{1\%}_{1cm}$ 250 nm of the saffron samples used in the validation.

Sample	Total crocetin glycosides (% on dry basis, mean ± SD)[a]	HPLC Picrocrocin (% on dry basis, mean ± SD)	Δ*E*pic (mean ± SD)	$E^{1\%}_{1cm}$ 250 nm
1	24.7 d,e ± 0.7	17.5 f ± 0.5	0.552 e,f ± 0.004	48.5 e,f ± 2.5
2	28.4 g,h,i ± 0.9	26.1 j,k ± 0.8	0.749 k ± 0.006	73.2 j,k ± 3.7
3	26.4 f ± 0.8	23.3 h ± 0.7	0.671 h,i ± 0.008	61.4 g,h ± 3.1
4	27.6 g ± 0.8	27.0 k ± 0.8	0.723 j ± 0.018	71.4 j,k ± 3.6
5	17.8 a ± 0.5	12.3 c ± 0.4	0.396 a ± 0.012	33.5 c ± 1.7
6	24.5 c,d ± 0.7	16.3 d,e ± 0.5	0.568 f ± 0.007	45.0 e ± 2.3
7	27.9 g,h ± 0.8	19.8 g ± 0.7	0.715 j ± 0.022	46.8 e ± 2.3
8	18.1 a ± 0.5	11.6 b,c ± 0.4	0.412 a ± 0.010	29.9 b,c ± 1.5
9	24.2 c ± 0.7	22.3 h ± 0.7	0.652 h ± 0.004	58.9 g ± 3.0
10	20.9 b ± 0.6	15.3 d ± 0.5	0.501 c ± 0.012	39.1 d ± 2.0
11	28.6 h,i,j ± 0.9	26.2 j,k ± 0.8	0.745 k ± 0.024	75.8 k ± 3.8
12	29.4 j ± 0.9	24.7 i ± 0.7	0.794 l ± 0.021	65.5 h,i ± 3.3
13	20.8 b ± 0.6	16.8 e,f ± 0.5	0.522 d ± 0.004	45.2 e ± 2.3
14	23.7 c ± 0.7	19.8 g ± 0.6	0.609 g ± 0.021	52.0 f ± 2.4
15	26.2 e,f ± 0.8	26.3 k ± 0.8	0.744 k ± 0.018	69.6 i,j ± 3.5
16	27.9 g,h ± 0.8	22.7 h ± 0.7	0.758 k ± 0.019	59.6 g ± 3.0
17	24.0 c ± 0.7	7.1 a ± 0.2	0.437 b ± 0.002	20.8 a ± 1.1
18	26.1 e,f ± 0.8	25.2 i,j ± 0.8	0.676 i ± 0.017	66.6 i ± 3.4
19	17.2 a ± 0.5	10.7 b ± 0.3	0.417 a,b ± 0.017	28.5 b ± 1.4
20	20.6 b ± 0.6	19.8 g ± 0.6	0.543 e ± 0.001	47.0 e ± 2.4

[a] The same letter in a column indicates non significant differences according to Duncan's test at the 0.05% level.

(Table 2) and those with the highest or the lowest $E^{1\%}_{1cm}$ 257 nm, samples 12 or 19 respectively (Table 1). In addition, these last two samples were also the samples having the highest or lowest $E^{1\%}_{1cm}$ 440 nm, showing the influence of crocetin esters in $E^{1\%}_{1cm}$ 257 nm. Although Corradi and Micheli (1979) proposed the measurement of saffron's bitter taste by means of Δ*E*pic (Eq. (1)), there was no agreement between the sample with the highest picrocrocin content (sample 4) and the highest Δ*E*pic (sample 12), nor between the sample with the lowest picrocrocin content (sample 17) and the sample with the lowest Δ*E*pic (sample 5) (Table 2). All these lacks of coincidence seemed to corroborate the effect of other components apart from picrocrocin in $E^{1\%}_{1cm}$ 257 nm and Δ*E*pic. Results of $E^{1\%}_{1cm}$ 250 nm are presented in Table 2 and, unlike $E^{1\%}_{1cm}$ 257 nm or Δ*E*pic, these results demonstrated a higher capacity of $E^{1\%}_{1cm}$ 250 nm to order the samples according to the content of picrocrocin determined by HPLC. Some exceptions in the relative order of the samples were found.

Moreover, the regression analysis of $E^{1\%}_{1cm}$ 257 nm, Δ*E*pic and $E^{1\%}_{1cm}$ 250 nm versus picrocrocin content (%) gave the equations:

$$E^{1\%}_{1cm}257\ \text{nm} = 1.49(\text{picrocrocin content},\%) + 58.66, r = 0.8964 \quad (5)$$

$$\Delta E\text{pic} = 0.02(\text{picrocrocin content},\%) + 0.21, r = 0.9241 \quad (6)$$

$$E^{1\%}_{1cm}250\ \text{nm} = 2.69(\text{picrocrocin content},\%) + 0.68, r = 0.9866 \quad (7)$$

showing the good results for $E^{1\%}_{1cm}$ 250 nm in relation to the other two parameters.

The proposed method showed a good selectivity to determine picrocrocin. Its corresponding peak at retention time 5.84 ± 0.03 min was practically the only one present in the chromatograms of the picrocrocin fraction obtained by SPE (Fig. 3).

As shown in Table 3, the method also had a good linearity with $r > 0.999$ for both HPLC and UV–vis analyses. Quantification with HPLC and UV–vis data was based on the calibration equations displayed. The HPLC calibration curve in water was used to quantify the picrocrocin by direct injection of a 0.5 g L^{-1} saffron extract while in the rest of determinations the sample underwent a 1/10 dilution before measuring. For that very reason its range was higher than in the rest of the calibration curves. The ε of picrocrocin

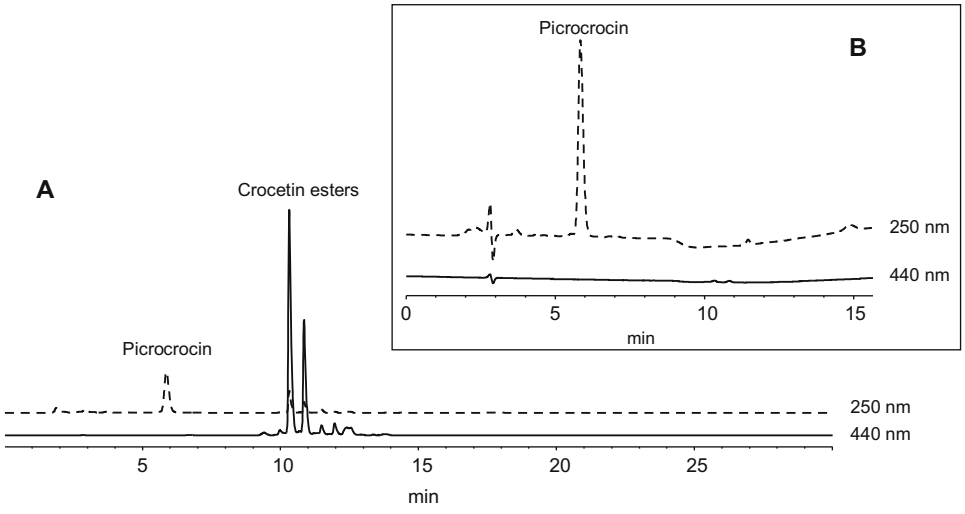

Fig. 3. Chromatograms at 250 and 440 nm of a saffron extract (A) and its corresponding picrocrocin fraction from SPE (B).

Table 3
Linearity of picrocrocin analysis by HPLC and UV–vis spectrophotometry.

	Regression curve data: picrocrocin concentration (mg L^{-1}) = ax[a] + b			
	Slope, a (mean ± SD)	Intercept, b (mean ± SD)	r	Linearity range (mg L^{-1})
HPLC (water)	0.0496 ± 0.0004	0.2709 ± 0.0667	0.9998	256.00–0.51
HPLC (acetonitrile 12%)	0.0453 ± 0.0001	0.1563 ± 0.0021	0.99995	60.00–0.60
UV–vis (water)	31.548 ± 0.193	0.030 ± 0.037	0.9998	51.20–0.51
UV–vis (acetonitrile 12%)	33.209 ± 0.200	0.123 ± 0.025	0.9998	60.00–0.60

[a] x = picrocrocin peak area in HPLC determinations or absorbance at 250 nm in UV–vis determinations.

obtained in acetonitrile/water 12% (v/v) was 9936 ± 113 L cm^{-1} mol^{-1} and totally agreed with the reported value of 9927 L cm^{-1} mol^{-1} (Sánchez, Carmona, Zalacain, & Alonso, 2005), whereas ε of picrocrocin in water was 10,482 ± 53, and also was in accordance with the value of 10,515 L cm^{-1} mol^{-1} reported by Sánchez et al. (2005) and the value of 10,100 L cm^{-1} mol^{-1} reported by Buchecker and Eugster (1973).

Although for routine quality control in the industry, our proposal is the expression of results as $E^{1\%}_{1cm}$ 250 nm, it was necessary for the results to be stated as % on a dry basis in order to compare the proposed method with the HPLC reference. With regard to HPLC data, it was only necessary to relate the picrocrocin concentration obtained through calibration curves with the saffron concentration in the extract. In the case of data from the proposed method, Eq. (4) could be applied. Recovery results ranged from 87.9 ± 1.8 for sample 9, to 93.1 ± 1.9 for sample 2 (Complete results are shown in the Supplementary Data). Taking into account the mentioned ε of picrocrocin in acetonitrile/water 12% (v/v) and the satisfactory recovery results of approximately 90%, therefore a factor of F = 1.1, Eq. (4) was applied as:

$$\%\ \text{of picrocrocin on dry basis} = \left(E^{1\%}_{1cm}250\ \text{nm}\right) \times 0.366 \qquad (8)$$

The comparison of means with the Student t test for checking the trueness resulted in no significant differences in the content of picrocrocin determined by both methods in any of the samples studied except in sample 7. In addition, the results from the two methods correlated significantly at a 0.01 level; $y = 0.9849x - 0.2647$, (y = % of picrocrocin determined with the proposed SPE method, x = % picrocrocin determined with HPLC method); r = 0.9866. If it was assumed that $E^{1\%}_{1cm}$ 257 nm and ΔEpic are only due to picrocrocin, their corresponding content of picrocrocin as % on a dry basis could be assessed with the ε obtained, but they would be clearly overestimated from $E^{1\%}_{1cm}$ 257 nm and sometimes over and sometimes underestimated from ΔEpic.

Neither origin nor commercial grade of saffron was found to differentiate method performance as exemplified in the case of recovery (Supplementary Data).

An RSD = 5.6% indicated that the repeatability of the procedure was satisfactory. From the repeatability standard deviation it was useful to calculate the L_r (Eq. (2)) which enabled the analyst to decide whether the difference between duplicate analyses of a sample, determined under repeatability conditions, was significant. Its value was 2.5 expressed as % picrocrocin on a dry basis and 7.6 expressed as $E^{1\%}_{1cm}$ 250 nm. Intermediate precision determined by different analysts on three separate days was also found satisfactory (RSD = 9.3%). The L_R was calculated according to Eq. (3) and indicated the same as L_r but under reproducibility conditions. Its value was 4.6 expressed as % picrocrocin on a dry basis and 13.7 expressed as $E^{1\%}_{1cm}$ 250 nm. These values found were in consonance with those reported by Sánchez (1996) with regard to repeatability and reproducibility of $E^{1\%}_{1cm}$ 257 nm: 2.8% and 8.5%, respectively. More recently, Lechtenberg et al. (2008) have reported values of 8.9% for repeatability and of 9.0% for intermediate precision in the analysis of picrocrocin by HPLC.

The proposed method also showed a good sensitivity, the LOD was 0.30 mg L^{-1} of picrocrocin (0.6% on a dry basis of saffron) and the LOQ was 0.63 mg L^{-1} of picrocrocin (1.3% on a dry basis of saffron). This value was approximately 100 times lower than the value reported by Lechtenberg et al., 2008.

4. Conclusions

The SPE procedure developed and validated in this study gave good results for determining the content of picrocrocin in saffron spice samples from UV–vis spectral information. The procedure was found to be accurate, reproducible and sensitive enough for this application in samples from different countries. Furthermore, its common points with the ISO determinations in saffron, the short time necessary to carry it out and its simplicity make the proposed procedure of particular interest for routine quality control in the industry. The results obtained suggest that this SPE method could even be included in the present ISO/TS 3632.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.03.039.

References

Alonso, G. L., Salinas, M. R., Garijo, J., & Sánchez, M. A. (2001). Composition of crocetin esters and picrocrocin from Spanish saffron (*Crocus sativus* L.). *Journal of Food Quality, 24*, 219–233.

Buchecker, R., & Eugster, C. H. (1973). Absolute configuration of picrocrocin. *Helvetica Chimica Acta, 56*, 1121–1125.

Carmona, M., Sánchez, A. M., Ferreres, F., Zalacain, A., Tomás-Barberán, F., & Alonso, G. L. (2007). Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: Comparative study of samples from different geographical origins. *Food Chemistry, 100*, 445–450.

Carmona, M., Zalacain, A., Salinas, M. R., & Alonso, G. L. (2007). A new approach to saffron aroma. *Critical Reviews in Food Science and Nutrition, 47*, 145–159.

Carmona, M., Zalacain, A., Sánchez, A. M., Novella, J. L., & Alonso, G. L. (2006). Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and Gardenia jasminoides fruits. Tentative identification of seven new compounds by LC-ESI-MS. *Journal of Agricultural and Food Chemistry, 54*, 973–979.

Corradi, C., & Micheli, G. (1979). Determinazione spettrofotometrica del potere colorante, amaricante ed odoroso dello zafferano. *Bollettino Chimico Farmaceutico, 118*, 553–562.

Escribano, J., Alonso, G. L., Coca-Prados, M., & Fernández, J. A. (1996). Crocin, safranal and picrocrocin from saffron (*Crocus sativus* L.) inhibit the growth of human cancer cells in vitro. *Cancer Letters, 100*, 23–30.

EURACHEM (1998). *The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics*. Teddington, Middlesex, United Kingdom: LGC. (<www.eurachem.org>).

Grigoriadou, D., Androulaki, A., Psomiadou, E., & Tsimidou, M. Z. (2007). Solid phase extraction in the analysis of squalene and tocopherols in olive oil. *Food Chemistry, 105*, 675–680.

Iborra, J. L., Castellar, M. R., Canovas, M., & Manjón, A. (1992). TLC preparative purification of picrocrocin, HTCC and crocin from saffron. *Journal of Food Science, 57*, 714–731.

ISO/TS 3632-1, 2 (2003). Saffron (*Crocus sativus* L.) Part 1: Specifications, Part 2: Test Methods. Geneva: ISO.

Lechtenberg, M., Schepmann, D., Niehues, M., Hellenbrand, N., Wünsch, B., & Hensel, A. (2008). Quality and functionality of saffron: Quality control, species assortment and affinity of extract and isolated saffron compounds to NMDA and σ1 (sigma-1) receptors. *Planta Medica, 74*, 764–772.

Lozano, P., Castellar, M. R., Simancas, M. J., & Iborra, J. L. (1999). Quantitative high-performance liquid chromatographic method to analyse commercial saffron (*Crocus sativus* L.) products. *Journal of Chromatography A, 830*, 477–483.

Orfanou, O., & Tsimidou, M. (1996). Evaluation of the colouring strength of saffron spice by UV–vis spectrometry. *Food Chemistry, 51*(3), 463–469.

Puoci, F., Curcio, M., Cirillo, G., Iemma, F., Spizzirri, U. G., & Picci, N. (2008). Molecularly imprinted solid-phase extraction for cholesterol determination in cheese products. *Food Chemistry, 106*, 836–842.

Rödel, W., & Petrizka, M. (1991). Analysis of volatile components of saffron. *Journal of High Resolution Chromatography, 14*, 771–774.

Sánchez, A. (1996). Evaluación de los métodos de la Norma ISO 3632 y los métodos SOIVRE para el análisis de azafrán mediante ensayo intercomparativo. In *Laboratorios del SOIVRE boletín técnico informativo. parámetros de calidad en el azafrán (I)* (pp. 39–45). Madrid: Lerko Print.

Sánchez, A. M., Carmona, M., Ordoudi, S. A., Tsimidou, M. Z., & Alonso, G. L. (2008a). Kinetics of individual crocetin ester degradation in aqueous extracts of saffron (*Crocus sativus* L.) upon thermal treatment in the dark. *Journal of Agricultural and Food Chemistry, 56*, 1627–1637.

Sánchez, A. M., Carmona, M., Zalacain, A., Carot, J. M., Jabaloyes, J. M., & Alonso, G. L. (2008b). Rapid determination of crocetin esters and picrocrocin from saffron spice (*Crocus sativus* L.) using UV–visible spectrophotometry for quality control. *Journal of Agricultural and Food Chemistry, 56*, 3167–3175.

Sánchez, A. M., Carmona, M., Zalacain, A., & Alonso, G. L. (2005). Aplicación de la extracción en fase sólida al análisis del poder amargo del azafrán especia. In *Avances de la ciencia y tecnología de los alimentos en los inicios del siglo XXI. Análisis de alimentos* (pp. 95–99). Burgos: Servicio de publicaciones Universidad de Burgos.

Sujata, V., Ravishankar, G. A., & Venkataraman, L. V. (1992). 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. *Journal of Chromatography A, 624*, 497–502.

Tarantilis, P. A., & Polissiou, M. G. (1997). Isolation and identification of the aroma components from saffron (*Crocus sativus* L.). *Journal of Agricultural and Food Chemistry, 45*, 459–462.

Tarantilis, P. A., Polissiou, M. G., & Manfait, M. (1994). Separation of picrocrocin, *cis-trans*-crocins and safranal of saffron using high-performance liquid chromatography with photodiode-array detection. *Journal of Chromatography A, 664*, 55–61.

Tarantilis, P. A., Tsoupras, G., & Polissiou, M. G. (1995). Determination of saffron (*Crocus sativus* L.) components in crude plant extract using high-performance liquid chromatography–UV–visible photodiode-array detection–mass spectrometry. *Journal of Chromatography A, 699*, 107–118.

Zalacain, A., Ordoudi, S. A., Blázquez, I., Díaz-Plaza, E. M., Carmona, M., Tsimidou, M. Z., & Alonso, G. L. (2005a). Screening method for the detection of artificial colours in saffron using derivative UV–vis spectrometry after precipitation of crocetin. *Food Additives and Contaminants, 22*(7), 607–615.

Zalacain, A., Ordoudi, S. A., Díaz-Plaza, E. M., Carmona, M., Blázquez, I., Tsimidou, M. Z., et al. (2005b). Near-infrared spectroscopy in saffron quality control: Determination of chemical composition and geographical origin. *Journal of Agricultural and Food Chemistry, 53*, 9337–9341.

Zarghami, N. S., & Heinz, D. E. (1971). The volatile constituents of saffron (*Crocus sativus* L.). *Lebensmittel-Wissenschaft und Technologie, 4*, 43–45.